Biocontrol efficacy of selected soil microbes

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By

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CERTIFICATE

This is to certify that the thesis entitled "**Biocontrol efficacy of selected soil microbes**" submitted by **Mrs. Sivakami .V** for the Degree of Doctor of Philosophy in Science of Madurai Kamaraj University, Madurai is based on the results of studies carried out by her under our guidance and supervision. This thesis or any part thereof has not been submitted elsewhere for the award of any other degree or diploma.

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DECLARATION

I hereby declare that the research work presented in this thesis entitled "**Biocontrol efficacy of selected soil microbes"** has been originally carried out by me under the guidance and supervision of **Dr. S. Kannan**, Professor & Head, Research Guide & Supervisor, Department of Environmental Studies, School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai, India. This work has not been submitted elsewhere for the award of any other degree or diploma.

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Sivakami.V

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ABBREVIATIONS

µl	microlitre
mg	milligram
g	gram
nm	Nanometer
^{0}C	degree Celsius
ml	milliliter
hrs	hours
mins	minutes
pH	hydrogen potential
EC	Electrical Conductivity
APHA	American Public Health Association
CFU	Colony Forming Unit
DNA	deoxyribonucleic acid
PCR	Polymerase Chain Reaction
EDTA	Ethylenediamine tetraacetic acid
Bp	base pair
BLAST	Basic Local Alignment Search Tool
MEGA	Molecular Evolutionary Genetics Analysis
KDa	Kilo Daltons
rpm	rotation per minute
ppm	parts per million
SEM	Scanning Electron Microscope
$FeSO_4$	Ferrous sulphate

BSA	Bovine Serum Albumin
SRBC	Sheep Red Blood Cells
SPSS	Statistical Package for Social Sciences
TAE	Tris-acetate-EDTA
PBS	Phosphate Buffer Saline
GPS	Glucose Peptone Salt
OD	Optical Density
HMDS	Hexamethyldisilazane
M	molarity

Introduction

1.0 Introduction

1.1 Environment and its components

> "The sum of all substances and forces external to an organism which determines its existence and regulates its process"
>
> \- Mason and Langenhim

The environment is the integrated system of both abiotic and biotic factors that influence with each other for better livelihood. The abiotic component consists of both physical and chemical characteristics of air, water and soil, whereas biotic components include living plants, animals and microorganisms.

The abiotic component, soil is a complex nature inhabited by many organisms including microorganisms such as bacteria, fungi, actinomycetes, algae, protozoa, *etc*. Among them, bacteria are unicellular, microscopic organisms that make up the largest part of the biomass and are responsible for the bulk decomposition in soil. According to Sylvia *et al.*, 1998, the number of different species or genomes per gram of soil estimated as a maximum of 10^6. There are two major categories of bacteria namely autotrophs which produce their food by utilizing inorganic salts and carbon dioxide for growth. In contrast, heterotrophs cannot make their food by themselves rather depend upon organic compounds by secreting enzymes to breakdown complex organic compounds to derive energy. Most of the soil bacteria are heterotrophs that are responsible for the degradation of biomass, take part in the biogeochemical cycle and provide nutrients to promote plant growth (Atlas and Bartha, 1998).

Certain plants like leguminous plants absorb the essential nutrients for their growth through its roots. During the developmental stage of plants, the secretion of various organic compounds through roots by exudation, which is called rhizodeposition. These compounds also act as a driving force for increased microbial growth in the rhizosphere region. Rhizosphere is defined as the region of soil adjacent to plant's roots especially in leguminous plants (Hiltner, 1904). The phenomenon of establishing micro flora in rhizosphere region under the influence of root is called the rhizosphere effect (Whipps, 2001). Hence, the rhizosphere is the hot spot zone for a large number of microbial colonization with high energy flux (Barrisu *et al.*, 2008).

The health of the soil in terrestrial ecosystem can be determined by the quantum of microbes present in the soil. Among the microbes, the bacterial population in the soil is mainly enumerated by using conventional culturing techniques like the plate count method in laboratory media. Recent advanced biochemical and molecular techniques are also used to enumerate bacterial population in the soil such as Phospholipid Fatty Analysis, Nucleic acid extraction, Polymerase Chain Reaction, r-RNA sequencing, Restriction Fragment Length Polymorphism, Amplified ribosomal DNA restriction analysis, cloning, sequencing techniques and microarrays (Butler *et al.*, 2003 and Teixeira *et al.*, 2010). The study stated that there are two major types of bacteria namely Gram-positive and Gram-negative which cover 15% of the total root surface in soil (Van Loon, 2007).

Several abiotic and biotic factors greatly influence the survival of soil microflora particularly bacterial population in rhizosphere soil. Abiotic factors such

as soil pH, salinity, texture, organic matter content, nutrient availability, seasonal effects and management practices like mode of irrigation, tillage, cropping, fertilizer and pesticide applications are reported as contributing factors which affect bacterial growth in rhizosphere soil (Fang *et al.,* 2005 and Ibekwe *et al.,* 2010). Biotic factors include the type of the plant, developmental stage of the plant, characteristics of root, plant defense mechanism which acts as major factors to determine their own rhizosphere bacteria (Mac Donald *et al.,* 2004).

Soil Rhizobacteria belongs to a group of bacterial population which promote plant growth. Antoun and Prevost (2005) reported that plant growth-promoting rhizobacteria (PGPR) occupies 2 to 5% of soil bacteria. This group of bacteria includes *Pseudomonas, Bacillus, Azospirillum, Azotobacter, Streptomyces, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Flavobacterium, Burkholderia, Bradyrhizobium, Serratia etc,* which enhance plant growth and control phytopathogens (Berg, 2000; Chen *et al.,* 2006 and Soltani *et al.,* 2010). *Pseudomonas* and *Bacillus* are extensively studied and commercially used for direct plant growth activities such as phosphate solubilization, siderophore production and release of phytohormones. These bacterial species are also indirectly helpful for plant health by producing toxins, extracellular lytic enzymes such as chitinases, proteases, cellulases against agricultural pests and public health pests like mosquitoes (Berg *et al.,* 2005).

1.2 Mosquitoes as a major concern in public Health problem

Mosquitoes are a large group of insects present throughout the temperate and tropical regions which belong to the family Culicidae and order Dipterans. They are considered as a nuisance pest that transmits vector-borne diseases to human beings and causes a major threat to the public health system. At present, a total of 3,540 mosquito species were recorded worldwide, categorized as two subfamilies and 112 genera (Harbach, 2014). In India, recorded mosquito population was about 393 species in which only 31 species are responsible for transmitting mosquito-borne diseases such as malaria, dengue, chikungunya, filariasis *etc* (Bhattacharyya *et al.*, 2014). Annually, vector-borne diseases account for 17% of all infectious diseases occurring worldwide, causing more than one million casualties. Recently, there is an increase in the transmission of vector-borne diseases gradually due to environmental and social factors which include globalization, unplanned urbanization without proper sanitation measures and climate change. According to WHO (2014), the epidemic potential of dengue leads to 30 fold increase in past 50 years and is considered a major challenge to the public health system.

During the 20th century, DDT was successfully used to control malarial transmission. But the frequent use of synthetic insecticides leads to the development of pesticide-resistant mosquitoes. The accumulation of pesticide residues in the environment causes bio-amplification in the food chain and produces undesirable effects on non-target populations such as earthworms, insects, frogs, aquatic birds, *etc*. Biological control is widely recommended as a desirable method due to its

benefits such as single application of biocontrol agent with long lasting effect, highly specific to pest, cost effective and harmless to other living organism in the environment (Hemingway and Bates, 2003).

1.3 Biological control

The biological control is defined as the control of the pest population by using natural enemies such as predators, parasites, pathogens, *etc*. A number of biological control agents formulated with bacteria, fungi, viruses, pheromones and plant extracts have been commonly used which differ in their pathogenicity and host specificity. Among these, bacteria capable of producing toxins, lytic enzymes, volatile compounds, *etc.*, which kill mosquitoes are termed as entomopathogenic bacteria (Porter *et al*., 1993 and Lacey *et al*., 2001)

Among the bacteria, several species of *Bacillus* are proved effective against different species of mosquitoes. Among all isolated strains of Bacilli worldwide, *Bacillus thuringiensis* serovar *israelensis* and *Bacillus sphaericus a*re highly potential to control breeding population of mosquitoes in order to maintain public health system (Lacey and Undeen, 1986).

In 1976, *B.thuringiensis* subsp. *israelensis* (*Bti*) was first isolated in Israel from dead insect and found toxic to mosquito larvae (Goldberg and Margalit, 1977). This bacterium was extensively studied and *B.israelensis* serotype (H-14) was found to be effective in the field registered for mosquito control in 1980. During sporulation, this bacterium produces crystalline inclusion bodies which act as inactive

protoxins. They were ingested by mosquito larvae and got solubilised in the insect midgut with alkaline (pH >10), leading to proteolytic activation. The activated toxins bind to receptors located on the midgut cell membranes and enters into membrane by forming pores that cause an osmotic imbalance and swelling of cells. This toxin paralyzes the insects gut and makes insects stop feeding, leading to larvae's death within few hours (Charles and Christina, 2000). Reports of this toxin against mammalian safety indicated that ingested and topically applied protoxin is not active against vertebrates (Shadduck *et al*., 1980).

Another Bacillus strain, *B.sphaericus* was first reported as mosquitocidal by Kellen *et al.,* (1965). The principal protein responsible for toxicity is the binary toxin, namely Bin toxin, produced during sporulation. The susceptibility of mosquito species to Bin toxin mainly depends on the receptor's presence on the midgut microvillar brush border membrane in mosquitoes. The toxin activity mainly occurs in midgut cells of mosquitoes, especially posterior stomach and gastric caeca. which were severely damaged by ingestion of toxin (Charles *et al.,* 1996). The persistence and prolonged residual activity of *B. sphaericus* in aquatic habitat remain higher than *B. thuringiensis israelensis* because the parasporal body of *Bti* assembles outside the exosporium membrane and dissociates from the spore after lysis, but the Bin toxin forms on the internal surface of this structure and remain associated with spore after lysis (Darboux *et al*., 2001).

More than 300 *B. sphaericus* strains have been isolated and identified worldwide (Baumann, 1991). *B. sphaericus* has been used effectively to control *Culex*

pipiens and *Culex quinquefasciatus* in aquatic habitat with high organic pollutants. Within a few years, *Culex species* remain resistant to single Bin toxin of *B. sphaericus* in China and Thailand (Yuan *et al*., 2000 and Mulla *et al*., 2003). Therefore, many genetic engineering techniques were used to clone genes encoding endotoxin proteins from *Bti* and *Bs* and generate new recombinant bacterial strains. These recombinant strains were highly effective than wild-type strains of *Bti* and *Bs* (Federici *et al*., 2003).

Most non-spore forming species of bacteria were isolated from insects belonging to family, *Enterobacteriaceae* or *Pseudomonidaceae*. These non-spore-forming bacteria generally occur as normal microflora in an insect’s digestive tract due to their low pathogenicity. They also become pathogenic when they have the ability to enter the insect's hemocoel and produce bacterial septicemia. These are collectively called as facultative pathogens (Lysenko, 1985). Studies reported that *Pseudomonas* species such as *Pseudomonas fluorescens* (Mikhnovskaia and Povazhnaia, 1975, Murty *et al*., 1994, Prabakaran *et al*., 2003, Varun and Pandian., 2008), *P.pseudomallei* (Lee and Seleena, 1990) and *P.aeruginosa* (Som *et al*., 1980 and Chadde, 1992) produce exotoxins active against different species of mosquitoes such as *Aedes*, *Anopheles*, *Culex* and *Culiseta* species. At Vector Control Research Centre (VCRC), Puducherry State, India, the microbial metabolite of *Pseudomonas fluorescens* Migula was identified and reported as active against larval and pupal stages of mosquito species. VCRC-B426, 0.09% emulsifiable concentrate (EC) formulation developed was tested for efficacy against *Culex quinquefasciatus* larvae,

and pupae also showed significant results in field trails. The pupicidal metabolite of VCRC-B426 strain was mass-produced and identified as rhamnolipid (Prabakaran *et al*., 2009). Reports also stated that *P. fluorescens* showed chitinase and gelatinase like activity against mosquito species (Mette and Jan, 1999 and Brammacharry & Paily., 2012).

Many researchers began the screening of bacterial strains from various ecological sources to isolate and identify novel strains. These novel strains were tested against mosquito species and the effective strains are used as a better alternative to existing bioinsecticides. Therefore, the present study was planned to carry out this work.

Objectives

2.0 Objectives

The present study was aimed

1. To determine the physicochemical characteristics of soil samples from agricultural fields and non-agricultural sites.

2. To isolate and identify bacterial strains from soil samples using biochemical characterization.

3. To test the nature of the pathogenicity of isolated bacterial strains against sheep blood cells.

4. To evaluate bacterial strain's biocontrol efficacy against larvae of mosquito vectors, namely *Aedes aegypti* and *Culex quinquefasciatus.*

5. To characterize the molecular phylogeny of highly effective bacterial strains using 16S r-RNA sequence analysis.

6. To determine the effects of toxicity in the morphology of mosquito larvae treated with mosquitocidal bacterial strains.

Review of Literature

3.0 Review of Literature

3.1 Soil as a substratum for microbes

Soil is a heterogeneous medium of solid, liquid and gaseous phases with varying properties both across the landscape and in-depth. It is considered the land surface of the earth which provides the substratum for both plant and animal life, including microorganisms such as bacteria, actinomycetes, fungi, algae and protozoa contribute to the maintenance of matter and energy turnover through a biogeochemical transformation in the terrestrial environment. The bacterial population in soil remains as one-half of the total microbial biomass in the soil which depends upon the physicochemical properties of the soil (Luo and Zhou, 2006). The majority of the bacterial population is concentrated in niches like rhizosphere zone that have a constant supply of easily utilizable nutrients by the plants. This act as a driving force for a group of the active and enhanced bacterial population in the root zone in higher volume. The bacterial load ranges from 10^{10} to 10^{12} per gram of soil in the rhizosphere (Grayston *et al.*, 1998 and Raajimakers *et al.*, 2009).

3.1.1 Factors influence bacterial diversity in soil

The bacterial population depends upon several abiotic and biotic factors of a particular ecological niche. In case of any small change in abiotic conditions including environmental stress and perturbation, soil and its rhizosphere bacteria sensitize and show response to it. Hence, they are considered as bioindicators of soil quality (Raaijmakers *et al*., 2002). Generally, soil parameters such as soil pH, soil

texture, organic matter content and concentration of nutrient elements are reported as the major factors affecting the bacterial composition of the rhizosphere.

3.1.2 Physicochemical properties of agricultural soil

Soil quality results from interactions between physical, chemical and biological properties in which microbes mediate reactions account for 80-90% of soil processes. Therefore, it is essential for a comprehensive assessment of physical, chemical and biological properties of soils need to explain the relationship between soil environment and microbes. Human activity, especially agriculture, influences the soil environment and its microbiome (Nannipeiri *et al.*, 2003).

The soil's pH is also one of the most important physicochemical parameter and differs from the type of ecosystem. It was observed that, the bacterial growth remains higher in neutral soils due to the availability of a wide variety of nutrients in this pH range whereas lower in acidic soils. All these soil parameters are inter-related to promoting soil productivity (Torsvik and Ovreas, 2002). Soil electrical conductivity mostly reflects the clay content, salt content and minerals which determine 80% of the soil's microbial groups. It also serves as a measurement of soluble nutrients and it is useful in monitoring the mineralization of organic matter in the soil.

Alvarez *et al.*, (2002) reported that the soil bacterial growth is greatly influenced by many soil parameters in which soil texture acts as an essential factor. Fine-textured soils (clay humic complex soils) typically contain organic matter

entrapped through adsorption and aggregation with a small pore size (<50 microns) and increases substrate utilization efficiency than coarse- textured soil (sandy soils). A study was conducted to evaluate bacterial community structure and diversities located in three different ecosystems such as agro, humid and forest ecosystems, situated in the same geographic area of Mascara (Northern –Algerian West) with soil and plant type. The bacterial count was significantly higher in the humid ecosystem and correlated with soil texture such as clay and silty clay ($P<0.005$) and no significant relationship was observed between fungi, actinomycetes and soil physico-chemical properties. Bacterial abundance displayed a positive linear relationship ($P = 0.005$) with soil pH and electrical conductivity. It also showed that soil organic matter was higher in the forest ecosystem but lower in agro ecosystem soils (Meliani *et al.*, 2012).

The importance of soil physico-chemical properties influencing soil bacterial communities from twelve locations in Sunderben mangrove forests, Bangladesh showed that soil pH, moisture, nitrogen content varied greatly among the studied locations. The total bacterial colony's enumeration reported higher in Koromjal site (14.5×10^4 CFU/g soil) than that in the Hironpoint site (7.65×10^4 CFU/g soil). There was no significant correlation among soil physico-chemical properties in the study area. Only soil total nitrogen (%) content showed positive correlation ($r = 0.718$, $P > 0.01$) (Hossain *et al.,* 2012).

Zhihua *et al.,* (2014) reported that physical and chemical properties of rhizosphere soil such as soil moisture content, pH, soil organic matter, total nitrogen

content, total phosphorus, total potassium, enzyme activities (sucrase, protease, urease and catalase) and microbial count was evaluated in soil samples collected from five bamboo forests. The rhizosphere soils in bamboo forests had significantly higher soil organic matter levels and available nutrients such as phosphorus and nitrogen content with lower pH than control soil. This showed that soil enzymatic activities and bacterial communities were considerably higher in bamboo forests than in barren land.

Laldinthar and Dkhar, (2015) reported that the importance of physicochemical properties in driving soil bacterial communities in the broadleaved forest stands of Meghalaya State was studied at two different altitudes such as Upper Shillong at higher altitude and Mawkyrdep at a lower altitude for two years. Results showed that bacterial count was higher in high altitude forest stand and positively correlated with moisture content($r = 0.70$; $p<0.05$), organic carbon($r=0.77$; $p<0.05$) and at low altitude forest stand positive correlation occurs with organic carbon only($r= 0.70$; $p =0.05$). A total of 14 bacterial species were isolated and *Bacillus subtilis* and *Microccocus* species were predominant. Both organic carbon content and total nitrogen act as major driving factors of bacterial communities in broad-leaved forest stands in Meghalaya State in North Eastern India.

The relationship between soil physico-chemical properties and soil bacterial community was observed for soil samples in the Sulaimaniyah governorate, Kurdistan region, Iraq. Correlation analysis showed that the bacterial population was significantly influenced with clay concentration ($r =0.90$), pH ($r=-0.85$), organic

matter (r=0.80), total nitrogen (r=0.81) and C/N ratio (r=0.92). Particularly, the organic carbon content ($p<0.05$) correlated with bacterial population in clay loam and slity clay loams compared to other type of soils. In this study, there was no significant correlation between fungal populations with soil physico-chemical properties (Mohammad, 2015).

Kamali *et al.*, (2017) reported that 65% of the correlation coefficients were positively correlated between bacterial counts and soil physico-chemical properties from soil samples in semi-arid parts in Central Sudan. Out of eight bacterial species isolated, six species were classified under *Bacillus* genera and two species under *Micrococcus* genera. Soil pH values in sandy clay loam and clay loam were observed as (7.24) and (7.78), respectively and the highest organic carbon content was recorded in sandy loam due to organic nutrients, which decrease with increase in soil depth.

Soil Organic Carbon (SOC) is associated significantly higher in clay-rich soils than coarse soils, which influences the growth and diversity among microbial populations. SOC is a key attribute in maintaining soil tilt and quality and energy source for microorganisms in soils. It also influences other soil functions, such as charge characteristics, aggregate stability and water holding capacity. Kumar and Rai, (2017) reported a strong positive correlation between phosphate solubilizing and organic carbon (r= 0.692) from paddy field soil samples taken from Indo-Gangetic plain in India. Gammaproteobacteria group of bacteria was highly abundant in rice agricultural fields which these bacteria can produce enzymes to degrade carbon substrates for their growth.

Hence, soil condition has been continuously altered by organic and inorganic components which profoundly affect the bacterial population. These above studies showed that agricultural practices could impact the bacterial population richness and diversity of soils through alterations in soil physical and chemical properties.

3.1.3 Physicochemical properties of non-agricultural soils

Sewage sludge is a residual mixture of organic and inorganic solids derived from municipal wastewater treatment. It contains a large amount of major and micronutrients besides having high organic matter content. Hence, it can improve soil physical, chemical and biological properties (Singh and Agrawal, 2009).

The sludge amended soil had more organic matter than the garden and bare saline soils, and this could account for the source of nutrients needed for microbial survival and multiplication. The high amount of organic carbon in sludge impacted soil corresponded to a high bacterial population, partly because soil organic carbon is a strong indicator of soil fertility status. It releases nutrients for plant growth, promotes the structure, biological and physical health of the soil and it is a buffer against harmful substances. Soils with large quantities of negative charge are more fertile because they retain more cations. The clay fraction has a more diverse bacterial community than silt or sand fractions. Thus, the sludge-impacted soil because it yielded more bacterial diversity related to high clay fraction.

Benzarti *et al.*,(2007) reported that the impact of the successive application of municipal solid waste compost on agricultural soils was investigated under field-scale

study for four consecutive years in Northern Tunisia. Results showed that increased numbers of total heterotrophic bacteria with increased soil enzyme activities were present in farm applied with municipal solid waste compared with farm applied with organic manure. Anthropogenic conditions greatly influence the soil condition by the releasing waste and its deposition on soil poses a more significant problem.

The impact of long-term integrated application of sewage sludge, compost and fertilizer nitrogen on microbial activity and soil health in rice rhizosphere of rice and wheat cropping system was evaluated and the maximum bacterial count was observed in treatment compared to control. The addition of organic inputs (compost and sewage sludge) favored bacterial activity. The decreased beneficial effect of sewage sludge may be due to the toxicity of undesirable materials as heavy metals present in sludge. The actinomycetes population increased due to high organic matter in sludge. The overall effect was observed as maximum through compost than sludge (Gill *et al.,* 2016).

Mehete *et al*., (2019) reported that samples from garden soil, saline soil and sludge-impacted soil were studied to evaluate the influence of soil properties on bacterial abundance and diversity. In sludge impacted soil showed the presence of *Actinobacteria* that play a vital role in the cycling of organic compounds linked with soil organic matter production, including manufacturing of the black pigments called melanin associated with soil humic acid. Members belonging to the *Firmicutes* phylum are dominant in saline and garden soils. Members of this group particularly

the genus *Bacillus* will be predominant in the sludge and their abundance is associated with their ability to resist desiccation.

3.2 Interaction between insect and soil bacteria

The bacterial population exhibits diverse interactions with many eukaryotes including insects. These interactions may be symbiotic, pathogenic and vectoring, *etc.* The bacterial cell starts with adhesion to the host (insect), invasion of the host, establishment and dissemination within the host by avoiding host immune responses and also concludes transmission either back to the environment or a new host (Sanchez-Contreras and Vilsidou, 2008). On the other side, insect physiology and susceptibility to infectious agents are highly affected by stress-inducing factors including food deprivation, mechanical forces and chemical poisoning by the presence of bacterial toxins that compromise immune defenses. Fine balance in the insect gut micro flora maintenance is essential for the optimal physiology in the insect (Ashburner *et al.*, 2005).

3.2.1 Entomopathogenic bacteria

Microorganisms that are pathogenic to insects and other arthropods are termed as entomopathogens, which include bacteria, fungi, viruses or protozoans. Entomopathogens contribute to the natural regulation of many populations of arthropods. The pathogenic effect of microbes on the target pests is so species-specific. Of all entomopathogens, bacteria have been the most extensively used organisms due to their desirable features such as shorter generation time, cost-effective mass production of bioinsecticides easy storage, and finds application in

fields with a different formulation such as emulsion, granular or dustable powders *etc* with long sustainable effects (Copping and Menn, 2000)

Among the bacteria, *Bacillus thuringiensis* (*Bt*) was discovered in Japan by Shigetane Ishiwata in the year 1901 as the causative agent of Sotto disease in silkworms. This is sporulating, Gram-positive, motile bacteria that produces crystal proteins. These toxins are exploited for control of agricultural pest but the control of dipteran species has been in practice only after the discovery of *B.thuringiensis* serovar *israelensis* (*Bti*) in 1977. Apart from soil, it can also be found in other environmental niches including phylloplane and insect host intestinal system, rarely causing natural epizootic episodes (Jensen *et al.*, 2003). *Bacillus thuringiensis* (*Bt*) spore-associated toxins are extremely virulent which can persist in the environment with high potency, specificity and covers the largest activity spectra including larvae from many *Culicidae* (mosquito) genera such as *Culex*, *Aedes*, and *Anopheles.* In contrast, they do not affect on vertebrates and non-target invertebrates (Miura *et al*., 1980).

3.2.2 Mode of action of bacterial entomopathogens

Bacterial entomopathogens or its toxins must be ingested and enter into the insect's alimentary tract where they multiply or activate to initiate disease. Mostly bacterial toxins and enzymes target midgut cells that disrupt the epithelial barrier and break through into the body cavity. This is followed by bacterial proliferation in the hemocoel, which causes septicemia, leading to the death of insects. Most of the toxins

produced by microbial pathogens are identified as peptides but vary significantly in structure, toxicity and specificity (Delecluse *et al*., 2000)

3.2.3 Bacilli group

In the case of *Bti*, Cry and Cyt toxins are four major crystal proteins which act synergistically against the mosquito population. The Cry (Cry4Aa, Cry4Ba, and Cry11Aa) and Cyt (Cyt1Aa) toxins are membrane-perforating proteins in which the Cry proteins bind to membrane receptors, whereas Cyt1Aa binds with high affinities to unsaturated phospholipids. These proteins have a greater affinity towards Diptera because of the higher proportion of unsaturated phospholipids in dipteran cell membranes than in other insects (Bravo *et al*., 2007)

Another member of the *Bacillus* genus's insecticide toxin-producing species is the *B. sphaericus,* which form the part of *B. subtilis* group. *B. sphaericus* is toxic against mosquito larvae and possesses two types of toxins: the highly active binary toxins BinA/BinB within spore crystals and the Mtx toxins. The target insect's binary toxins are ingested and get solubilised, proteolytically activated, bind specific receptors and form pores in target cells disrupting the mosquito gut epithelium (De Maagd *et al.*, 2003 and Hu *et al.*, 2008).

B.cereus can be occasionally found in the insect gut not only as spores but also as growing vegetative cells. While lacking δ-endotoxin genes, the *B. cereus* genome carries various genes that cause disintegration of insect tissues and produces an immune inhibitor protein (InhA). This protein specifically hydrolyses cecropins and

attacins in the immune hemolymph of *Hyalophora cecropia* in vitro condition (Helgason *et al*., 2000 and Fedhila *et al.*, 2003).

Nadarajah *et al.*,(2006) reported that the role of carbohydrates on the toxic effect of parasporal inclusion proteins isolated from Malaysian mosquitocidal *B. thuringiensis* (Bt) strains on erythrocytes (human and rat) was determined that *Bt* toxin is a lectin which recognizes specific plasma membrane glycoconjugate receptor with a terminal residue of either D-mannose, N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc) or combination of these monosaccharides.

Ramasamy *et al.*, (2008) reported that the bioactivity of vegetative proteins from uncharacterized Malaysian *Bt* (seven strains) and *Bs* (two strains) against *Ae.aegypti* and *Cx quinquefasciatus*. Except for *Bt* 17 and *Bt* 21, vegetative proteins from all *Bt* and *Bs* strains were highly hemolytic to human erythrocytes, which cause more than 75% hemolysis at highest concentration of 200 µg/ml. Vegetative proteins from *Bt* strains shown the activity against both species of larvae but vegetative proteins from *Bs* were weakly larvicidal to *Cx. quinquefasciatus* only.

The biosurfactant production was identified from strain VCRC B483 *Bacillus amyloliquefaciens* isolated from soil samples collected from the mangrove forests of Andaman and Nicobar Islands. Optimization studies showed that biosurfactant production from this strain was increased with incubation time and maximum biomass. This was tested against larvae and pupae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* following WHO standard protocols. The larvicidal

and pupicidal activity was found effective against *An.stephensi* compared to *Cx.quinquefasciatus, Ae.aegypti.* The biosurfactant's lipoprotein nature was confirmed by β hemolysis, lipase activity, biofilm forming capacity and thermostability. The mosquitocidal biosurfactant produced by *Bacillus amyloliquefaciens* (VCRC-B483) act as prospective molecule (Geetha *et al.,* 2014).

Lysinibacillus sphaericus, formerly *Bacillus sphaericus* was renamed due to lysins and aspartic acid in the composition of their peptidoglycan. These are heterogeneous group of Gram- positive sporulating *Bacillus* entomopathogenic against mosquito larvae. Purified S-Layer from both *Lysinibacillus sphaericus* C7 and 2362 strain culture was toxic against *Culex sp*. larvae. In vitro assay using mimic substrates (chitin compounds and sheep red blood cells) showed that these strains possess hemolytic activity and Chitin binding activity, which act as an essential feature for insect pathogenicity (Allievi *et al.,* 2014)

Later, Bashir *et al*., (2016) reported that *Bacillus laterosporus* isolated from a commercial formulation showed that larvicidal activity against second instar larvae of *Ae. aegypti*, *An.* s*tephensi* and *Cx. quinquefasciatus*. The toxin was heat stable and its toxicity increased with inoculum size and incubation time. Rajendran *et al*., (2018) reported that *Bacillus thuringiensis* was isolated from rhizosphere soil collected from *Bt* cotton field. Crystal (cry) proteins were extracted and tested for mortality rate of the mosquito larvae. Among the tested isolates, RBL10 and RBL20 showed that highest percentage of larvicidal activity at 96% and 83% respectively.

3.2.4 *Pseudomonas* group

The bacteria that are used as biopesticides can be divided into four categories: crystalliferous spore formers (*B. thuringiensis*), obligate pathogens (*Bacillus popilliae*), potential pathogens (*Serratia marcesens)* and facultative pathogens (*Pseudomonas aeruginosa*). It was reported that *Pseudomonas entomophila* closely related to *Pseudomonas putida* act as an entomopathogenic bacterium, which can infect and kill *Drosophila melanogaster* upon ingestion. This bacterium secretes a strong diffusible hemolytic substance, which is related to the production of a new cyclic lipopeptide containing 14 amino acids and endolysin with strong biocontrol activity (Vallet Gely *et al.*, 2010 and Koul, 2011).

The mosquitocidal activity of extracellular proteins of *P. fluorescens* was demonstrated using chitinase assay. The effect of purified protein in the alimentary canal and gut region of the treated larvae and pupae of *Cx. quinquefasciatus* was studied and found to exhibit chitinolytic activity and degraded the cuticular proteins in the treated larvae and pupae of *Cx. quinquefasciatus.* The mode of action behind chitinase toxicity is the enzymatic hydrolysis of chitin, a common constituent of the insect exoskeleton and gut lining of the peritrophic membrane. It leads to disruption of ionic regulation and death of the larvae or pupae treated with the proteins (Usharani and Paily, 2012 and Suganthi *et al*., 2017).

The extracellular polysaccharide was characterized from *P. aeruginosa* B01. 1H NMR spectra of EPS extracted from *P. aeruginosa* B01 exhibited characteristic chemical shifts (ppm) and corresponding functional groups. Acid hydrolysis of EPS

was carried out using sulfuric acid and yielded hydrolyzed product of polysaccharide monomers. The purified EPS showed potent activity against mosquito larvae (Benit and Roslin, 2018).

Ertruk and Yaman, (2019) reported the isolation of five non-spore forming bacterial strains from the European Cockchafer, *Melolontha melolontha* and was found effective against three economically important pests belongs to orders Coleoptera and Lepidoptera of Arthropod Phyllum. The bacteria were identified as *Enterobacter cloacae, Serratia marcescens*, *Pseudomonas aeruginosa*, *Kocuria kristinae*, *Serratia liquifaciens*. Among these strains, *Pseudomonas aeruginosa* was found highly active against larvae with an 84% mortality rate.

3.3 Biocontrol efficacy of soil bacteria

Biological control is the use of the non-chemical and environmental friendly method of controlling insect pests by natural control agents such as parasites and predators. Though chemical insecticides remain successful in eliminating vector populations, increasing reports of high levels of chemical insecticides residues in edible fruits, vegetables, groundwater and even mother's milk lead to the disappearance of honey bees and other rare fauna (Chandrasekaran *et al.*, 2016). There are three types of biological control strategies applied in pest control programs, namely Importation, Augmentation and Conservation. Primarily, Importation is also referred as classical biological control which is defined as the intentional introduction of an exotic (non-native) usually co-evolved biological control agent for the permanent establishment and long-term control (Van Driesche, 2008). Secondly,

Augmentation involves the supplemental release of natural enemies boosting the naturally occurring population. Thirdly, Conservation biological control is defined as the modification of the environment or existing practices to protect and enhance specific natural enemies of other organisms to reduce the effect of pests through habitat manipulation approaches (Sanda and Sunusi, 2014).

In 1996, WHO provided guidance on laboratory studies, small scale and large scale field to determine efficacy, field application rates, operational feasibility and acceptability of mosquito larvicides. This helps to harmonize the testing procedures carried out in different laboratories and institutions to generate data for registration and labeling of larvicides by national authorities (WHO, 2005). Hence, many researchers continuously screen microbes from different environmental habitats to identify key compounds to act as potential candidate larvicides that can be mass produced and commercially available in the market.

3.3.1 Screening of Bacteria for mosquitocidal activity

The screening for microorganisms for mosquito larvicides was examined in the guts of mosquito larvae collected from natural breeding habitats of *Ae. aegypti* and *Cx. quinquefasciatus* larvae. *Bacillus* species such as *Bacillus cereus* were found to be dominant bacterial species in their guts. The relationship between these *Bacillus* strains and the mosquito larvae was determined by introducing the bacteria into larvae of *Ae. aegypti, Cx. quinquefasciatus* and another common mosquito strain, *An. dirus.* The cell numbers of *Bacillus cereus* strains AelO and Cx5 in the guts were consistent throughout seven days period without food supplementation showed that these strains

were able to colonize in the larvae's guts of the larvae. Under similar conditions, *B. thuringiensis* serovar *israelensis nc4Q2-72* was hardly detectable after two days, while *Escherichia coli* could not be detected at all. Their stable retention in mosquito larvae guts and genetic manipulation's feasibility indicate these strains possess high potential as novel host cells for application in mosquito control (Luxananil *et al.,* 2001).

A new strain of *Bacillus circulans* was isolated from a larva of *Cx. quinquefasciatus,* which showed larvicidal activity on medically important mosquitoes. Compared with *B. sphaericus* strain 2362, *B. circulans* isolate provided less toxicity to *Cx. quinquefasciatus* and *An. gambiae* and possess 107 times more toxicity to *Ae. aegypti*. *B. circulans* was proved as pathogenic as *B. thuringiensis var. israelensis* against *Ae. aegypti*. The tests have showed that the toxicity of *B. circulans* resulted from its spores and not from the insecticidal effect of chitinases or exotoxins (Darriet and Hougard, 2002).

Park *et al.,* (2009) reported that two spore forming bacteria, M413 and C32 were isolated from sediment samples collected in Manatec County, Florida. Lyophilized powder of sporulated cultures of M413, C32 and *B. thuringiensis* subsp. *israelensis* IPS-82 were tested against mosquito *Ae.aegypti, Cx.quinquefasciatus, Oc. taeniorhynchus* and *An.quadrimaculatus.* Results showed that M413 is mainly toxic to *Oc.taeniorhynchus* (LC_{50} = 89.2 ng/ml) whereas C32 is (LC_{50} = 82.2 ng/ml) to *Cx quinquefasciatus.* M413 was not active against *Ae. aegypti* and *Cx quinquefasciatus* but showed low toxicity against *An.quadrimaculatus* ($LC_{50=}$1155 ng/ml). C32 did not

show any toxicity against *Ae.aegypti* or *An.quadrimaculatus* but showed low toxicity against *Oc.taeniorhyncus* (LC_{50} = 739.2 ng /ml). But none of these isolates showed higher toxicity against any mosquito species than *B.thuringiensis subsp.israelensis* IPS – 82.

Screening assay of ten microbial isolates was used for larvicidal activity. Five were active against mosquito larvae which were identified as *B. megaterium, B. sphaericus, B. cereus, B. subtillis* and *Acinetobactor* sp. under laboratory conditions. The two isolates (*B. megaterium* and *Acinetobactor* sp.*)* were considered as most toxic strains followed by *B. sphaericus, B. cereus,* and *B. subtillis* with LC_{90} values 4.1 ± 0.39, 2.8 ± 0.17, 3.6 ± 0.37, 2.5 ± 0.71, 3.6 ± 0.71 mg/ml respectively under laboratory conditions and the mortality rate was 97% at 48 hrs exposure tests. This study showed that non-spore formers of common microbial isolates from the natural environment were also able to kill the larvae of *Aedes aegypti* (Radhika *et al.*, 2011).

Patil, 2012 reported that two native bacterial isolates, *Bacillus thuringiensis* (*Bt* SV2) and *Serratia* sp. (SV6) were evaluated for mosquito larvicidal potential against the early fourth instar larvae of *Ae aegypti*, *An. stephensi* and *Cx. quinquefasciatus* with reference to *B. thuringiensis* subsp. *israelensis (Bti)* H 14. *Bt* SV2 isolate showed 100% mortality against early fourth instars of *Ae. aegypti*, *An. stephensi,* and *Cx. quinquefasciatus,* similar to the *Bti* H14 strain. After 24 h, *Bt* SV2 showed 98%, 89%, and 80.67%, and *Bti* H14 showed 92%, 98.33%, and 60% mortality against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus,* respectively.

Serratia SV6 showed the highest activity against *Cx. quinquefasciatus* (100%) followed by *An. stephensi* (95%) and *Ae. aegypti* (91%) after 48 h of exposure. The Gram-negative *Serratia* SV6 showed delayed toxicity compared to *Bti* H14 and *Bt* SV2 against early fourth instars of *Ae aegypti*, *An. stephensi*, and *Cx. quinquefasciatus* and it was considered as the first report on mosquito larvicidal potential of *Serratia* species.

Ramathilaga *et al.*,(2012) reported that two bacterial species isolated from dead mosquito larvae were identified as *Peanibacillus macerans* and *Bacillus subtilis* was examined for their mosquito larvicidal activity against chikungunya vector, *Ae.aegypti.* The LC_{50} values of *P. macerans* and *B. subtilis* were recorded 70.99 and 58.97 24 hrs and 48 hrs respectively. The LC_{50} value of the procured culture *Bacillus thuringiensis* subsp *israelensis* also detected as 152.02 for 24 hrs and 48 hrs. Hence, *Ae. aegypti* was the most susceptible to *B. subtilis* with the highest relative susceptibility (RS) value.

González *et al.,* (2013) reported that an extensive screening of 150 soil samples was collected and screened for mosquitocidal activity from soil samples in Western Cuba. The protein profiles of the crystal components were determined by SDS-PAGE. Eight hundred and eighty-one bacterial isolates were obtained and among 881 bacterial isolates, only 13 isolates with entomopathogenic activity were isolated from nine samples. These isolates showed that higher entomopathogenic activity against both *Cx. quinquefasciatus* and *Ae. aegypti* compared with the

reference strain 266/2. These isolates showed a protein pattern similar to the *B. thuringiensis* var. *israelensis* IPS-82 and 266/2 strains.

Poopathi *et al.,* (2014) reported that mosquitocidal bacteria were screened from excreta of wild birds discharged on the phylloplane of trees of garden situated at the premises of vector control Research Centre, Puducherry State in India. Out of 1000 samples examined, twelve bacterial strains were identified as mosquitocidal and 16 SrRNA analysis showed that these isolates belonged to *Bacillus* species (*Bacillus thuringiensis*, *Bacillus sphaericus* and *Bacillus cereus*). Toxicity assay showed that *Bacillus sphaericus* VCRC-B547 (NCBI:JN377789) remain highly active against *Cx. quinquefasciatus, An. stephensi* and *Ae. aegypti*. The SDS-PAGE protein analysis revealed that a considerable difference in the protein profiles at the regions of 20-200 KDa. This suggested that interrelationship exists between wild birds with occurrence of mosquitocidal bacteria.

The toxicity of a novel bacterial product of soil bacterium, *Pseudomonas frederiksbergensis* isolated from contaminated soil in Saudi Arabia was tested against 3rd instar larvae of filarial vector, *Culex pipiens*. This active compound was identified as glycolipid which act as a potent mosquito larvicide. The larvicidal bioassay showed that LC_{50} and LC_{90} values were 434.45µl/l and 767.50µl/l respectively after 24-hour treatment (Ahmed *et al.,* 2014).

Balakrishnan *et al.,* (2015) reported that a mosquitocidal bacterium was isolated from the mangroves of Vellar estuary, South East coast of India. The soil and

water samples were collected from the mangroves habitat and bacterial strains were screened against mosquitocidal activity against mosquito larvae of *Ae. aegypti* and *An. stephensi*. Among the four isolated strains, such as *Bacillus cereus, Bacillus subtilis, Bacillus thuringiensis and Bacillus sphaericus,* three strains (*B.subtilis, B.thuringiensis* and *B.sphaericus)* showed potential mosquitocidal activity. The mortality range was about 50-75% within 24 hrs and was gradually increased between 4-92% after 48 hrs treatment.

Vinodhkumar *et al.,*(2015) reported that a total of 15 marine Actinomycetes were isolated from Mullakadu saltpan which is situated Tuticorin coast region of Tamil Nadu. Of the fifteen screened isolates of actinomycetes, only five isolates showed anti-larval activity against *Cx. quinquefasciatus, Ae. aegypti* and *An. stephensi* . Only three isolates showed potential to inhibit (66.6%) the growth of mosquito larvae. ISO7 and ISO11 showed the potential to inhibit the growth of the *Culex quinquefasciatus, Aedes aegypti* and *Anopheles stephensi* larvae whereas ISO2 showed the least capable to inhibition (33.3%) of the mosquito growth.

The efficacy of two different species of *Pseudomonas fluorescens* (NCIM 2099 and NCIM 2100) metabolites was studied against third instars larvae of *Aedes aegypti* and *Culex quinquefasciatus* mosquito larvae. The liquid formulation was prepared from metabolites of *Pseudomonas fluorescens* which was lethal to mosquito larvae. Results indicated that NCIM 2099 metabolite exhibit 94.4% and 92% larvicidal activity on *Ae. aegypti,* while NCIM 2100 exhibit 92% and 64% larvicidal activity on *Cx. quinquefasciatus* (Vankudre *et al.,* 2015).

Nabar and Lokegankar (2015) reported that 86 samples were collected from different extreme environments such as hot water springs of Vajreshwari in Maharashtra, Kargil desert, extremely cold regions of Sikkim, Srinagar and forest regions at higher altitudes of Kodaikanal and Chikhaldara. Screening of samples showed that 124 bacteria were isolated totally in which 24 are psychrophiles, 38 are mesophiles and 63 are thermophiles. From hot spring regions of Vajreshwari, four *Bacillus* spp. isolates were effective against *Culex* larvae at LC_{50} of 100ppm. The forest regions of Chikhaldara yielded thermophiles such as *Corynebacterium* and *Bacillus* sp. and Psychrophilic *Pseudomonas* sp. were isolated from Sikkim whereas Kodaikanal soil showed mixed a population of Psychrophiles, thermophiles and mesophiles. These isolates were screened against larvae of mosquito species such as *Ae. aegypti* and *Cx. quinquefasciatus*. Preliminary screening results showed that 21 bacterial isolates were effective against both species. Secondary metabolites were extracted from these bacterial isolates, and a sub-lethal concentration of 16 isolates showed that 100% mortality at 500 ppm concentration. SKP2 and SKT1 showed that maximum toxicity towards *Aedes* mosquito larvae with 80% and 70% respectively. In *Culex* larvae, LC_{50} was from 100 ppm to 400 ppm as 100% in 24 hrs. CKT2, KT1 and KP2 showed maximum efficacy against *Culex* sp. larvae in 24 hrs. Secondary metabolites from *Pseudomonas* sp. were effective against *Ae. aegypti* larvae while *Bacillus* was also effective against *Cx. quinquefasciatus* larvae.

Mahamuni *et al.,* (2016) reported that strains of *Pseudomonas fluorescens* (NCIM -2631) and *Pseudomonas caryophilly* (NCIM -5094) were screened to find

their ability to produce the toxin to inhibit mosquitoes larvae. Results showed that both strains have ability to produce extracellular exotoxin which exhibit mosquitocidal activity against *Ae. aegypti*. The exotoxin produced by the *Pseudomonas fluorescencs* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM - 5094) was partially purified and also confirmed its mosquitocidal activity after purification.

Lalithambika and Vani (2016) reported that bacterial isolates KUN1, KUN2, KUN3, KUN4 and KUBS were isolated from agricultural field's rhizosphere soil from Coimbatore, Tamil Nadu. Lyophilized culture supernatants of KUN2 (24, 48, and 72 hrs) culture and solvent extracts from the diethyl ether, petroleum ether, chloroform and ethyl acetate were tested against the fourth instar larvae of *Ae aegypti.* Morphological and biochemical characterization of KUN2 showed that *Pseudomonas* spp. Molecular methods confirmed it as *P.aeruginosa*. Toxicity studies revealed that culture supernatant of KUN2 (24 and 48 hrs) showed more toxicity than other isolates towards *Ae. aegypti* larvae. The highest mortality rate was recorded as 100% at 24 hrs when treated with 100 µg/ml of petroleum ether extract of KUN2.

3.3.2 Field testing of mosquitocidal bacterial agents

The first commercial product of *Bt* "Sporeine" was introduced in France in 1983. In continuous research in search of new entomopathogens, there was gradual development of several products with high virulence and stability. In this regard, various biological control agents have been thoroughly investigated with the support

of United Nations Development Programme/ World Health Organization Special Programme for Research and Training in Tropical Diseases (WHO/TDR). Certain strains of bacteria, especially *Bacillus thuringiensis* var *israelensis* (*Bti*) and *B. sphaericus* have been found to be highly effective for the control of larvae of mosquitoes and some other dipterans. These bacterial agents have been developed as larvicides, which are commonly known as biocides or biolarvicides. During past two decades various biolarvicide formulations produced in India and abroad have been tested at Malaria Research Centre (MRC), and some of these formulations have undergone large-scale operational trials. The efficacy of *B. sphaericus* and *Bti* preparation against mosquito larvae depends on the formulation which suite with the biology and habitat of the target mosquito species (Mittal., 2003).

3.3.3 Factor affecting field efficacy of biolarvicides

The efficacy of bacterial preparations against target mosquitoes is greatly influenced by various physicochemical and biotic factors such as temperature, pH, sunlight, sedimentation rate of spores, organic pollution, larval stage, density, *etc* (Mulla, 1985 and Becker *et al*., 1992).

Temperature is an important factor which influences the toxicity of these bacterial preparations. The efficacy of spherix (*B. sphaericus)* especially against anopheline larvae was greatly reduced in laboratory bioassays at 21^{o}C as compared to 31^{o}C which indicated that biolarvicide will not be effective in colder region but remain effective in tropical climate conditions in India (Chatterjee *et al.,* 2008). Similar results were also obtained with bactoculicide (*Bti*) formulation but with a

lower degree of difference. In addition, pH of the environment (>10) greatly reduced the activity of spherix (*B. sphaericus*) and bactoculicide (*Bti*) against larvae of *An. stephensi* in laboratory bioassays. Protein toxins of *Bti* and *B.sphaericus* and highly sensitive to UV radiations from sunlight which reduces the activity of biolarvicides.

Various other factors such as presence of organic particulate matter, stage and number of larvae and the type of biolarvicide formulation (with respect to surface or bottom feeding behaviour of larvae) also influences the activity of bacterial preparations in laboratory bioassays. The residual efficacy of biolarvicides in field conditions also influenced by the type of formulation, vegetation and organic pollution which affect the sedimentation rate of spore toxin of biolarvicide. Generally, aqueous suspension or flowable liquid formulation of biocides produced better results against column feeding *Culex* species whereas dust formulations or surface spreading formulations remain more effective against surface feeding *Anopheles* species.

The granular and tablet formulations were more effective against *Ae. aegypti* (Mittal., 2003). The *Bti* formulations have been commercialized by many companies namely Sandoz (Teknar ®), Abbot (Vectobac ®), Solvay (Bactimos) which were used on a large scale in the bio-control of mosquitoes and blackflies in many countries (de Barjac, 1990).

3.4 Molecular identification of entomopathogenic bacterial isolates

Due to the limitation in the biochemical approach for species identification of bacteria that remain tedious, expensive and inaccurate in species-level identification,

there was a need for a rapid and definitive method. Yamamoto and Harayama, (1995) studied nucleic acid fingerprinting, especially the PCR- based methods, has been widely applied in bacterial systematics. Use of 16SrRNA genes in molecular systematics provides two primary advantages over phenotypic identification: one is rapid turn-around time and another is improved accuracy. The information content of the 5' end of the 16SrRNA gene is sufficient to identify most bacterial species. The 16SrRNA sequences contain two types of the region, a highly conserved region that define the relationship among distant taxa and a variable region which differentiates the genera and species.

3.5 Mechanisms of mosquitocidal activity

Electron microscopy has developed knowledge about ultra-structure of the cells and tissues. It helps to determine molecular structure, interactions and processes including structure-function relationships at cellular level with resolution in atomic to nano-metre range. SEM permits non-destructive evaluation of the specimen and also very short specimen preparation time only few seconds in which the specimen is attached to a “stub” (specimen holder). Large specimens of about 200 mm diameter wafers or even larger can be used in SEM. Knowledge of the cellular ultrastructure contributes to an understanding of how cells and tissues function in both normal physiological and pathological state.

Compared to light microscope, SEM offers a better resolving power in observing fine biological specimens (Hollenberg and Erickson, 1973). The detailed information of the morphological description gained interest to researchers for

taxonomical identification. Hence, SEM is frequently used to observe the external morphology and fine structure of mosquito eggs (Linley, 1989, Service and Linley, 1997 and Matsuo *et al*., 1972) and adults (Bowen, 1995) of *Culicidae* family.

Raj *et al.*, (2012) reported that a modified method for acquiring micrograph of the fine structure of the fourth instar larvae of *Aedes aegypti* and *Aedes albopictus* was demonstrated using the Scanning Electron Microscope (SEM). The method developed did not utilise any fixation agents such as glutaraldehyde or osmium tetroxide but only ethanol (as dehydration agent). The electron micrographs showed that intact segment of both the *Aedes aegypti* and *Aedes albopictus* larvae.

Ahmed, (2013) reported that about the histological damages in the gastric caeca have been demonstrated in third instar larvae of the autogenous *Aedes caspius* upon infection with *Bacillus thuringiensis* (*Bt*). Both control and *Bt*-infected larvae were observed under SEM for investigating the body size (in terms of head and thorax widths) alterations at 40 h post-infection. SEM investigation revealed that the size of thoraxic region was considered as an indication of the body size alteration which showed that a significant shrinkage in the thoraxic region of infected larva compared to control ones (untreated). Thorax of *Bt*-infected larvae showed a significant 50% reduction in size compared to that of the control ones. However, head capsule was similar in both infected and control larvae. This clearly showed that a significant shrinkage in the body size of *Bt*-infected larvae.

The toxicity of a novel bacterial product of *Pseudomonas frederiksbergensis* isolated from contaminated soil in Saudi Arabia was tested against filarial vector, *Culex pipiens*, mosquito. Scanning electron microscopy revealed morphological symptoms of toxic effect of *P.frederiksbergensis* extract which showed that external shrinking of larval cuticle in treated larvae. Moreover, a significant shrinkage in the whole body size was detected in treated larvae compared to control in 24 hrs post-treatment. In addition, the histological studies with light microscopy showed that *P. frederiksbergensis* extract cause destructive effects on treated larvae mid-gut epithelial cells with cytoplasmic extensions by cellular and nuclear degradation followed by peritrophic membrane devastation that lead to septicemia. During eight hrs post treatment, larvae ceased feeding resulting in starvation and death at 24 hr post-treatment (Ahmed *et al.*, 2014).

Kirti and Shipali, (2014) reported that the scanning Electron Microscope was used to determine the morphological aspects of immature stages (fourth instar larva and pupa) of *Anopheles stephensi* to evaluate additional taxonomic features such as number of teeth on mentum, shurt and long processes on Pecten and shape of trumpet were found which cannot be possible in normal identification procedure.

Bryopsis pennata is a seaweed species under the family of *Bryopsidaceae* which was widely distributed in tropical and temperate marine waters. In this study, the mosquitocidal activity of the extracts of seaweed *Bryopsis pennata* was evaluated against dengue vectors *Aedes aegypti* and *Aedes albopictus.* The Chloroform extract exhibited strong ovicidal activity (with LC_{50} values of 229.3 and 250.5 μg/ml) and

larvicidal activity against *Aedes aegypti* and *Aedes albopictus*. In addition, SEM investigation revealed that larvae treated with chloroform extract of *B. pennata* were observed to have darkened body segments and shrunken anal papillae as compared to the control larvae. It was clearly reported that the treated larvae had spiracular apparatus with damaged inner structures (Ke-Xin *et al.*, 2015).

Krishnan *et al*., (2020) reported that the cellular and tissue damage of *Aedes aegypti* and *Culex quinquefasciatus* larvae was caused by selenium based nanoparticles using *Dillenia indica*. The visual damages include cell lysis, breakage of peritrophic membrane and pre-rupturing stages of epithelial cells. At increased concentration of 100 ppm, tissues of larvae treated with SeNps showed that completely disorganized and broken epithelial cells. These effects also include damages in the internal organs like broken midgut, caeca and totally collapsed larvae.

The above literature survey indicates that soil microbes has an impact on insects population and the studies have been conducted on regular basis in various areas to identify the best candidate to control insect pests of both agricultural and medical importance. There are many lacunae in the bionomics and utilization of soil microbes. In this context only, the present study has been planned, conducted and reported

Materials and Methods

4.0 Materials and Methods

Based on the objective to evaluate the biocontrol efficacy of selected soil microbes, the study was designed and the soil samples were collected in Madurai district.

4.1 Description of the sampling area

The soil samples were classified into different sites which consist of both agricultural and non-agricultural sites. The agricultural sites include paddy field, banana plantation, sugarcane field, bamboo plantation, vegetable farm and cattle fodder farm with sewage sediment as manure. The non-agricultural sites include irrigation canal, pond sediment and sewage sediment site on drainage as shown in (Fig.41).

4.1.1 Collection of soil samples from different sources

The rhizosphere soil samples from agricultural fields were collected by removing the sub-surface soil. Using a sterile spatula, two to four cm depth of surface soil which remains adjacent to roots were aseptically collected and transferred into sterile polythene bags. The sediment soil samples from non-agricultural sites were also collected aseptically using the sterile spatula and transferred into sterile polythene bags. They were brought to the laboratory at Department of Environmental studies, Madurai Kamaraj University, Madurai and stored at 4^0C for further analysis.



Fig 4.1 Location of soil sampling sites from different environmental sources

4.2 Physicochemical characteristics of soil samples

The physical, chemical and biological properties of soils are interrelated with each other. Any change in these soil parameters will directly influence the soil quality, which in turn affects plant growth and microbial activity. Therefore, it is essential to study the physico-chemical properties of soils periodically to promote soil

productivity. In this present study, the collected soil samples from identified various sampling sites were subjected to analyze physico-chemical characteristic features adapting standard methods (APHA, 2012).

4.2.1 Estimation of soil pH

Soil pH is a measurement of the concentration of ions bound to soil particles and organic matter. It is a physical parameter directly affects nutrient availability in soil, plant growth and microbial activity. The soil pH was determined using digital pH meter. The pH meter was calibrated using pH- 7 buffer solution. Then the meter was adjusted with the known pH valued buffer solutions namely 4.0 and 9.2. Each soil sample of 20g was weighed and mixed with 40 ml of distilled water in a sterile beaker both soil and water in the ratio of 1:2. This soil suspension was allowed to stand for half an hour with intermittent stirring. The electrode was immersed in soil suspension, and the pH value was measured (Dewis and Freitas, 1970).

4.2.2 Estimation of Electrical Conductivity

Electrical conductivity (EC) is a measurement of soluble salts present in soil suspension to conduct electrical current through it. EC is measured in units called Seimens per unit area (mS/cm or milliSeimens per centimeter). The conductivity measurements should be made within a few hours of preparation of soil suspension. Electrical conductivity can be measured using a meter and probe. The probe consists of two metal electrodes spaced 1 cm apart and it was calibrated using a standard solution of known conductivity. For that, 0.01 M of Potassium chloride was prepared as a reference solution with electrical conductivity of 1.413 dS/m at 25^0 C. 10 g of air-

dried soil was mixed with 50 ml of deionized water in the ratio of 1:5. It was mechanically shaken for 15 rpm for one hour to dissolve the soluble salts. The probe tip was calibrated by immersing it into a reference solution to obtain cell constant. Then the probe was rinsed with deionized water and immersed into soil suspension without disturbing settled soil particles. The current flowing through the sample is directly proportional to the concentration of dissolved ions in the sample (Rayment and Higginson, 1992). The Electrical conductivity (EC) was calculated using the formula

EC (ds /m)= S x 1.413/K

Where S=Measured EC of suspension

K=Measured EC of Potassium chloride solution

1.413=Electrical conductivity of reference solution

4.2.3 Estimation of organic carbon content

The Walkley - Black (WB) titration method is one of the classical methods for rapid analysis of organic carbon (OC) in soils and sediments. This method is based on the oxidation of organic matter by Potassium dichromate –sulfuric acid mixture by ferrous ammonium sulfate

One gram of air-dried soil was transferred into a 500 ml conical flask.10 ml of 1N Potassium dichromate solution and 20 ml of concentrated sulfuric acid was added into the flask and mixed thoroughly. The mixture was allowed to stand for 30 min. for the completion of the reaction. 200 ml of distilled water was added to the flask to

dilute the suspension.10 ml of Orthophosphoric acid and 1 ml of diphenylamine indicator were added to the flask, and the solution colour changed to deep violet. It was titrated with 0.5 N Ferrous ammonium sulfate until color changes from violet to blue and finally bright green. The volume of the ferrous ammonium sulfate used in the titration was noted. A blank titration without soil was made to standardize Ferrous ammonium sulfate solution against Potassium dichromate solution (Walkey and Black, 1934).

Carbon content in soil (%) = (B-T) X S X 0.003 X 1.3 X 100/W

Where B=Amount of $FeSO_4$ required in blank titration

T=Amount of $FeSO_4$ required in soil titration

S=Strength of $FeSO_4$ (from blank titration)

W=Weight of the soil.

Organic matter in soil (%) = % Organic C X 1.724

4.3 Correlation analysis (Zar, 2009)

In statistics, correlation is defined as the measure of the relationship between two variables. The correlation coefficient (r) is a quantitative assessment that measures both the direction and the strength of the relationship between two variables, whether it is positively correlated or negatively correlated. The coefficient (r) values less than 0.8 or greater than -0.8 are not considered significant. The correlation between physico-chemical properties of soil samples and the bacterial count was analyzed using MS-Excel software. The correlation coefficient values of

each parameter and its significance level were calculated to find out the positive and negative correlation between the variables.

4.4 Isolation of bacterial strains from soil samples

Pure culture techniques were performed to isolate and characterize bacterial strains from different environmental sources. The soil samples were collected after removing the surface soil and samples were taken using a sterile spatula from a depth of 2 to 5 cm below the surface. The collected samples bagged in sterile polythene bags were brought to the laboratory in Department of Environmental studies, Madurai Kamaraj University and stored at 4°C on refrigerator (Cappuccino and Sherman, 2001).

4.4.1 Enumeration of bacterial colonies

The soil suspension was prepared by adding one gm of the soil sample in a conical flask containing 10 ml of sterile distilled water and agitated for one hour. One ml of sample was taken which was serially diluted in sterile test tubes up to 10^6 dilution factor from this soil suspension. 0.1ml of aliquot of each dilution 10^5 and 10^6 were poured onto the nutrient agar plates to isolate bacterial colonies. Each colony-forming unit (CFU) represents the number of viable cells in a sample. CFU of a sample determined by multiplying the number of colonies on a dilution plate by the corresponding dilution factor. CFU /ml = (no. of colonies x dilution factor)/volume of culture plate (Collins and Lyne, 1980).

4.5 Biochemical characterization of bacterial strains

The isolated bacterial colonies were subjected to identify biochemical characteristic features which includes Gram's staining, motility test, catalase test, oxidase test, Nitrate reduction test, Gelatin hydrolysis test, Indole test, Methyl Red test (MR) and Voges Proskauer test (VP) according to standard microbial techniques. Each sample's pure culture was stored in agar slants, and each bacterial isolate was given specific codes for future reference.

4.5.1 Gram's staining test

This staining method is essential to differentiate bacteria into Gram-positive and Gram- negative bacteria. A loopful of overnight bacterial culture was smeared onto a glass slide and heat-fixed by rapidly passing the slide two to three times on Bunsen burner. The smear was flooded with crystal violet solution for one min. Then, the slide was washed with tap water and flooded with lugol's iodine for one min. Again the slide was washed with water, blot dried and decolorized by washing in a gentle stream of 95% ethyl alcohol for 30 sec to remove excess stain. Counterstaining was done by flooding with safranin for 20 sec. The slide was again washed with tap water and blot dried. The smear was observed under compound microscope under different magnifications (Cappuccino and Sherman, 2001).

4.5.2 Motility test

Motility test was performed with overnight culture. In a cavity slide, one drop of bacterial broth culture was placed in the centre. A coverslip was placed on the top

of the cavity slide and it was observed under the microscope to analyze their motility study.

4.5.3 Catalase test

A loopful of overnight bacterial culture was placed onto a clear grease-free glass slide. One drop of 3% hydrogen peroxide was dropped on the culture and observed for production of gas bubbles. The presence or absence of bubbles indicated whether catalase-positive or catalase-negative.

4.5.4 Oxidase test

The oxidase test identifies bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. In the presence of oxidase enzyme, it oxidases the reagent tetramethyl-p-phenylenediamine into indophenols with purple color formation as an end product. In the absence of the enzyme, the reagent is reduced and remains colorless. 4.5 ml of nutrient broth was inoculated with bacterial culture and incubated for 24 hrs. To this, 0.2 ml of 1% α-naphthol and 0.3 ml of 1% p-aminodimethylaniline oxalate (Gaby and Hadley reagent) was added. Followed by the test tube was shaken vigorously for thorough oxygenation of the culture and observed for dark purple in colour.

4.5.5 Nitrate reduction test

This test is used to determine the ability of an organism to produce Nitrate reductase enzyme. Nitrate broth was prepared and inoculated with bacterial culture to be tested. The inoculated broth tubes were incubated at 37^0C for four hrs. Then, added

a few drops of reagent A (sulfanilic acid) and reagent B (α-napthylamine) observed the colour change due to the reduction of nitrate to nitrite (Conn and Breed, 1919).

4.5.6 Gelatin hydrolysis test

This test is used to determine an organism's ability to produce gelatinase (proteolytic enzyme) which liquefy gelatin. This is helpful in identifying and differentiating species of *Bacillus, Clostridium, Proteus, Pseudomonas,* and *Serratia*. The test tube containing gelatin was stabbed with overnight bacterial culture and incubated along with an un-inoculated tube at 37^0 C for two weeks. The tubes were tilted to observe the hydrolysis of gelatin (Greene and Larks, 1955).

4.5.7 Indole test

The indole test is used to demonstrate certain bacteria's ability to decompose the amino acid tryptophan to indole. This test is essential in the identification of Enterobacteria. In a sterile test tube, 4 ml of tryptophan broth prepared was poured and inoculated with 24 hrs old bacterial culture. The tubes were incubated at 37^0C for 24 hrs. 0.5 ml of Kovac's reagent was added into the broth culture. The presence of red colour ring on the surface due to the reaction of para-Dimthylaminobenzalehyde with indole in the medium indicated positive result and yellow colour showed negative result (Benson, 2002).

4.5.8 Methyl red test

Methyl red test is used to detect bacteria's ability to produce stable acids end products from glucose as a carbon source. The sterile test tube containing MR broth

was inoculated with 24 hrs old bacterial culture and incubated at 37^0C. After incubation, five drops of methyl red reagent was added into the broth. In this test, methyl red act as pH indicator which changes to red colour in the presence of acid in the medium and yellow colour in the absence of acid. Results were observed as red colour in the positive reaction whereas yellow colour in the negative reaction.

4.5.9 Voges Proskauer test

Voges Proskauer test is used for the identification of Enterobacteriaceae. Bacteria metabolize glucose to key intermediate, pyruvic acid which can be further metabolized to produce acetoin. The sterile test tube containing VP broth was inoculated with 24 hrs old bacterial culture. This tube was kept for incubation at 37^0 C. After incubation, 0.6 ml of α-Naphthol reagent and 0.2 ml of 40% potassium hydroxide and tubes were shaken gently for 30 sec. The development of pink colour or red colour at the surface of the medium indicates the production of acetoin while yellow or copper colour at the surface of the medium indicates the absence of acetoin production.

4.6 Evaluation of bacterial extracellular proteins

The bacterial extracellular proteins were subjected to hemolytic assay to determine the toxicity of bacterial strains. Hemolysis of red blood cells is the measure to evaluate bacterial strain's ability to cause infection in target hosts. It also acts as the measuring index for assessing the entomopathogenicity of various bacterial strains isolated from different sources.

4.6.1 Enrichment of broth culture

The isolated pure cultures were transferred into the nutrient broth. After overnight incubation, one ml of culture broth was transferred into 50 ml of broth. Then, the bacterial culture was enriched by inoculating 50 ml culture into 100 ml of nutrient broth. The culture filtrate was obtained by centrifugation at 12,000 rpm for 10 min. The culture supernatant was transferred into sterile test tubes and stored at 4^0C (Wilson and Walker, 1994).

4.6.2 Estimation of total protein concentration in culture filtrate

The protein concentration in the culture supernatant was estimated by using Lowry's method. Different dilutions of Bovine Serum Albumin (BSA) was prepared by mixing stock BSA solution (1 mg/ml) and distilled water in the test tube. The final volume in each test tube is 5 ml. The BSA range is 0.05 to 1mg/ml. From these dilutions, 0.2 ml of culture filtrate solution was pipetted out to different test tubes and two ml of alkaline copper sulphate reagent (analytical reagent) was added and mixed thoroughly. The solution was incubated at room temperature for 10 min. Then, 0.2 ml of Folin Ciocalteau reagent solution was added and incubated for 30 min. The readings were observed in a spectrophotometer at 660 nm. The absorbance of an unknown sample was determined for absorbance of standard solution using the standard curve (Lowry *et al*, 1951).

4.6.3 Partial purification of proteins in the culture filtrate of bacterial strains

Ammonium sulfate precipitation technique was adapted to precipitate out water soluble proteins. These ions have a stabilizing effect on proteins and it does not

denature proteins. The bacterial culture (48 h) grown in Glucose Peptone Salt broth (GPS) was transferred into sterile centrifuge tubes and centrifuged at 8000 rpm for 15 min. Proteins in the culture supernatant was precipitated using ammonium sulfate (50%). 1/3rd of ammonium sulfate was added to the solution and allowed to dissolve. After that, the remaining portion of ammonium sulfate was added and placed in ice bath for 15 min. Then the tubes were centrifuged for 10 min at 10,000 rpm. The supernatant was discarded and white precipitate in the centrifuge tube was dissolved in sterile water and kept in the freezer for further use (Plummer, 1971).

4.6.4 Micro-hemolytic assay

The micro hemolytic assay was performed in 96 well 'V' bottomed microtitre plate by serially diluting 1% erythrocytes. Sheep blood was drawn from the jugular vein with a sterile syringe. It was then immediately transferred into a conical flask containing Alsever's solution (Sheep blood: Alsever's solution, 1:4) and stored at 4^0C. 1% Sheep blood (SRBC) was prepared by washing sheep blood (stored in Alsever's solution) thrice in physiological saline by centrifugation at 3000 rpm for 10 min. The pelleted/packed SRBC was resuspended in physiological saline to the required concentration in terms of percent packed SRBC, volume in the suspension. 50 µl of the crude toxin was serially diluted in the microtitre wells upto 11th well. 25 µl of 1% erythrocytes was added in all the wells and the plate was gently shaken for efficient mixing of reagents.

The microtitre plate was incubated at room temperature for an hour. 1% Triton-X was used as a positive control and physiological saline with S-RBC was

used as blank. The results were observed as uniform red colour suspension in the well showed that positive hemolysis as button formation at the bottom of wells shows negative hemolysis. The Optical Density (OD) values were observed in a spectrophotometer.

The percentage of hemolysis (%) =OD value of the sample-OD value of blank/OD value of 100% lysis tube-OD value of blank x 100 (Rowe and Wech, 1994).

4.7 Bioassay of extracellular proteins isolated from bacterial strains

Biocontrol efficacy of bacterial strains was determined by calculating the larval mortality rate against different culture filtrate concentrations obtained from bacterial strains. WHO stated certain guidelines to perform the laboratory experiment for evaluating the mosquitocidal activity of isolated bacterial strains from different sources. This protocol differs from plant extracted compounds to bacterial compounds.

4.7.1 Collection and rearing of mosquito larvae

Egg rafts of *Aedes aegypti* and *Culex quinquefasciatus* were collected from the Centre for Research in Medical Entomology (CRME), field station of Vector Control Research Centre (VCRC), Puducherry State in India. The eggs were reared in the plastic tray containing chlorine-free tap water in the laboratory with yeast tablets as feed. The water was changed once a day to avoid any microbial infection. The stages

of larvae were distinguished and separated in different trays for bioassay study (Asahina, 1964 and Gerberc, 1970).

4.7.2 Larvicidal activity of bacterial strains against mosquito vectors

The desired concentrations were obtained from culture filtrate and were used as test concentrations for bioassay against mosquito larvae. For every 100 ml of the test solution, 20 larvae were used. A control set along with three experimental sets, were also maintained. The larvae's mortality were observed after 24 hrs and 48 hrs, and the mortality of treated groups were corrected by using Abbott's formula.

Mortality (%) = (X-Y)/X x100. Where X = percentage survival in the untreated control and Y = percentage survival in the treated sample (WHO, 2005 and Abbott, 1925).

4.7.3 Regression analysis

In statistics, regression analysis was used to estimate relationships between a dependent variable and one or more independent variables. It helps to understand how the typical value of the dependent variable changes when any one of the independent variables is varied while the other independent variable remains constant. It can be utilized to assess the strength of the relationship between variables. Both dependent and independent variables shows a linear relationship between the slope and the intercept. A valuable numerical measure of association between two variables is the correlation coefficient, a value between -1 and 1 that indicates the strength of the association of the observed data for the two variables. The observed data of

concentration of exoproteins (X) and mortality rate (Y) was analyzed using the SPSS programme (Abbott, 1981).

4.8 Identification of bacterial strains by molecular characterization

The bacterial identification was performed by using the pure culture techniques and biochemical characterization. Recent molecular methodology to study bacterial phylogeny and taxonomy include genomic DNA isolation, Polymerase Chain Reaction and 16S ribosomal based on r-RNA identification was made. The 16S r-RNA gene sequences act as the most common genetic marker for bacterial identification due to this conserved region's presence in almost all bacteria with less variation.

4.8.1 Genomic DNA isolation of bacterial strains

Genomic DNA of bacterial isolates was isolated using Qiagen DNeasy DNA extraction kit. Two ml of sterile Eppendorf tubes were taken and labeled for each bacterial sample. To this, 1.75 ml of bacterial culture was transferred and centrifuged at 20,000 rpm for five min. Culture supernatant was decanted and 180 µl of enzymatic lysis buffer (ATL buffer) was added and vortexed for 10-20 sec. The tubes were incubated at 37^0C for 30 min. To this, 25 µl of proteinase K and 200 µl of AL buffer was added and vortexed. Then the tubes were incubated at 56^0C for 30 min. Again, 200 µl of 100% ethanol and 200 µl of AL buffer was added and vortexed. Using a micropipette, the entire contents were transferred to a labeled spin column and centrifuged at 8,000 rpm for one min. The column was removed from the collection tube and placed in a new collection tube. To this, 500 µl of AW1 buffer

was added and centrifuged at 8000 rpm for one min. The column was removed from the collection tube and placed in a new collection tube. 500 µl of AW2 buffer was added to the column and centrifuged at 14000 rpm for three min. The column was carefully transferred to a sterile 1.5 ml Eppendorf tube and 300 µl of MilliQ was added to it. It was allowed to stand at room temperature for one min. Again the tube was centrifuged at 10000 rpm for one min. The column was discarded and DNA was stored at 4^0C for short term storage and -20^0C for long term storage (Wang *et al*, 2011).

4.8.2 Agarose gel electrophoresis

Agarose gel electrophoresis is a common and highly effective method of separating, identifying and purifying DNA fragments. First, agarose gel was prepared with agarose concentration appropriate for the size of DNA fragments for separation. DNA samples are loaded into sample wells and the gel ran at a voltage for optimal separation of DNA fragments. Then, the gel was stained with ethidium bromide and visualized directly upon illumination with UV light. Agarose (1%) was prepared with (Tris-Acetate-EDTA) TAE buffer and melted in a microwave oven to dissolve the agarose in the buffer. The agarose solution was cooled down to 50^0C for five min. The solution was then poured into a gel tray with a well comb and left at room temperature for 30 min for complete solidification.TAE buffer was poured into the gel electrophoresis tank. The DNA sample (100 to 200 ng) was mixed with one µl of 6x dye and added into wells. The electrophoresis was started by switching on the DC powerpack. As the bromophenol blue (the tracking dye) has moved one cm above the

bottom end, the power supply was disconnected. The gel and the platform were stained in the plastic tray containing 0.5 µg/ml ethidium bromide in the sterile distilled water. After about 30-45 min, the platform and gel was rinsed with distilled water. The gel was observed through a gel documentation unit to visualize the bands (Sambrook *et al*, 1989).

4.8.3 PCR amplification of 16S r-RNA gene

Primers 8F and 1522 R were purchased and diluted with Milli Q water to make 100 µl of stock concentration. For this, 495.6 µl of MilliQ and 484.4 µl of Milli Q water were added to 8F and 1522 R primers, respectively. From this, 90 µl of Milli Q and 10 µl of 8F primer and 10 µl of 1522 R primer, to make working concentration.

The PCR reaction volume was made into 10 µl with of Master mix (2x) – 5 µl, 8F Primer (10 micromolar concentration) - 1 µl, 1522 R Primer (10 micromolar concentration) - 1 µl and Genomic DNA- minimum 100 ng required - 1 µl. In case of DNA less than 100 ng, 2 µl of Milli Q was added (Innis *et al*., 1990).

4.8.4 Purification of PCR products

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore) was used for 16S r-RNA sequencing (Brtelli and Greub, 2014).

4.8.5 16S r-RNA Sequencing

The PCR product was sequenced using the 27F/1492R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle

Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

The 16S r-RNA sequence was blasted using the NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed, followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar, 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignmentnoise) (Talavera and Castresana 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown as accurate as other existing phylogeny programs using simulated data while being one order of magnitude faster. PhyML was shown as accurate as other existing phylogeny programs using simulated data. The program Tree Dyn 198.3 was used for tree rendering (Dereeper *et al*., 2008).

4.9 Scanning electron microscopy (SEM) analysis of mosquito larvae

The scanning electron microscope (SEM) was used for the observation of specimen surfaces. When the specimen was irradiated with a fine electron beam, secondary electrons are emitted from the specimen surface. The specimen's topography was observed by two-dimensional scanning of the electron probe over the surface and an image is obtained from the detected secondary electrons. This microscopy requires an electro-optical system to produce an electron probe, a specimen stage to place the specimen, a secondary-electron detector to collect secondary electrons, an image display unit and an operation system various functions. The inside of the electron optical system and the specimen chamber was kept at a high vacuum of 10^{-3} to 10^{-4} Pa (Ratnayake *et al*., 2012).

4.9.1 Processing of larval samples for SEM observation

The third instar *Aedes aegypti* and *Culex quinquefasciatus* mosquito larvae of both control and treated (24 hrs treated) were prepared and observed for morphological changes in their body parts under Scanning Electron Microscopy (SEM). Both larvae were immediately fixed in 2.5% glutaraldehyde in 0.1 M Phosphate Buffer Saline. After 24 hrs of fixation, larvae were rinsed with 0.1 M Phosphate Buffer Saline three times at 10 min intervals and again fixed in 2% osmium tetraoxide in distilled water. Larvae were then rinsed three times in 0.1 M PBS and dehydrated in ascending series of ethanol (30%, 50%, 70% and 95%) for 10 min each and again rinsed three times in 100% ethanol. Finally, larvae were rinsed twice in 100% HMDS for 15 min and allowed to dry overnight. The processed

larval samples were further dehydrated to observe under SEM (Goldstein and Yakowitz, 1975).

4.9.2 Examination of morphological changes in treated larvae

Larval samples were dried using a critical point dryer. Then, it was fixed onto double-adhesive carbon tape and observed under the SEM. The resulting images from control and treated larval preparations were adjusted for contrast and suitable qualities.

Results

5.0 Results

5.1 Physicochemical properties of soil samples collected from different sampling sites

In the present study, soil characteristic features of collected soil samples were analyzed following standard methods. Pure bacterial colonies were isolated in nutrient agar using serial dilution followed by adapting spread plate techniques. The bacterial colonies were enumerated and expressed as colony forming unit per gram of soil (CFU/g). Among all these sampling sites, three paddy rhizosphere soil samples showed higher bacterial growth (0.8 $x10^9$ CFU/g, $0.6x10^9$ CFU/g and $0.5x10^9$ CFU/g) followed by two sugarcane plantation rhizosphere soil samples ($1.8x10^8$ CFU/g and $1.6x10^8$ CFU/g), two banana plantation rhizosphere soil samples ($1.5x10^8$ CFU/g and $0.8x10^8$ CFU/g) samples from agricultural field, garden soil ($1.5x10^8$ CFU/g), vegetable farm soil ($0.5x10^8$ CFU/g), cattle fodder farm soil ($0.4x10^8$ CFU/g) and bamboo plantation rhizosphere soil ($0.2x10^8$ CFU/g) as shown in Table 5.1.

Table 5.1 Physicochemical characteristics of soil samples from agricultural fields

S.No	Samples	Soil sampled location	Soil Type observed	Soil pH obtained	Electrical conductivity (μS)	Organic carbon content (%)	Nutrient agar (Hi media) CFU/g
1.	Site 1	Garden soil	Sandy loam	6.5	0.03	5.00	$1.5x10^8$
2.	Site 2	Paddy rhizosphere	clayey	6.5	0.10	10.00	$0.5x10^9$
3.	Site 3	Bamboo rhizosphere	sandy	6.2	0.04	1.20	$0.2x10^8$
4.	Site 4	Banana rhizosphere	Slity clay	6.5	0.12	6.50	$1.6x10^8$
5.	Site 5	Banana rhizosphere	Slity clay	6.7	0.08	7.00	$1.5x10^8$
6.	Site 6	Paddy rhizosphere	clayey	6.7	0.15	13.00	$0.6x10^9$
7.	**Site 7**	**Paddy rhizosphere**	**clayey**	**6.8**	**0.16**	**15.00**	**$0.8x10^9$**
8.	Site 8	Vegetable farm	Slity clay	7.1	0.65	4.50	0.5×10^8
9.	Site 9	cattle fodder farm soil	Slity clay	7.2	0.80	4.00	$0.4x10^8$
10.	Site 10	Sugarcane rhizosphere soil	Clay loam	6.5	0.07	9.00	$0.6x10^8$
11.	Site 11	Sugarcane rhizosphere soil	Clay loam	6.5	0.06	9.00	$0.8x10^8$

In non-agricultural field, where soil sampling sites selected such as sewage sediment soil, mosquito breeding site, pond sediment and irrigation canal site, the samples were collected and their characteristic features were analyzed using standard APHA methods. Among the nine samples of non-agricultural sites studied, only sewage sediment soil sample reported as having higher bacterial growth ($2.5x10^9$ CFU/g) followed by mosquito breeding site (1.5×10^9 CFU/g), irrigational canal side by soil (0.3×10^8 CFU/g) and pond sediment (0.1×10^8 CFU/g) (Table 5.2).

Table 5.2 Physicochemical characteristics of soil samples from non-agricultural fields

S.No	Samples	Soil sampled location	Soil Type observed	Soil pH obtained	Electrical conductivity (µS)	Organic carbon content (%)	Nutrient agar (Hi media) CFU/g
1.	Site 12	Mosquito breeding site sediment	loamy	6.5	0.18	16.00	$1.0x10^{9}$
2.	Site 13	Mosquito breeding site sediment	loamy	6.7	0.25	18.00	$1.5x10^{9}$
3.	Site 14	Mosquito breeding site sediment	loamy	6.8	0.20	20.00	$1.2x10^{9}$
4.	Site 15	Pond sediment	Sandy loam	7.4	0.18	1.6	$0.2x10^{8}$
5.	**Site 16**	**Sewage sediment**	**loamy**	**7.2**	**0.28**	**32.00**	**$2.5x10^{9}$**
6.	Site 17	Irrigational canal sediment	sandy	7.0	0.20	3.00	$0.3x10^{8}$
7.	Site 18	Irrigational canal sediment	sandy	7.1	0.05	2.00	$0.2x10^{8}$
8.	Site 19	sewage sediment	loamy	7.5	0.25	25.00	$2.0x10^{9}$
9.	Site 20	Pond sediment	Sandy loam	7.2	0.02	1.50	$0.1x10^{8}$

5.1.1 Correlation coefficient of agricultural field soil samples

The correlation between the soil physicochemical properties and the bacterial count was determined using SPSS software. Table 5.3 indicated that there was a significant correlation between bacterial colony counts with soil physicochemical properties. Among these soil parameters of agriculture field, only organic carbon content was correlated with bacterial count at the significance level (0.01). Both pH and electrical conductivity were negatively correlated with bacterial count. Total bacterial count was positively correlated with organic carbon content (r = 0.030; P = 0.930) and negatively correlated with pH (r = -0.148; P= 0.664) and electrical conductivity(r = -0.375; P = 0.256).

Table 5.3 Correlation coefficients between and within soil physicochemical properties of agricultural field samples and bacterial growth (df =20)

Parameters	pH	Electrical conductivity (dS m-1)	Organic carbon content(g kg^{-1})	Bacterial count on Nutrient Agar(Hi media) CFU/g
pH	1			
Electrical conductivity (dS $m^{-1)}$	r = 0.894** P = 0.000	1		
Organic carbon content (g kg^{-1})	r = 0.036 P = 0.916	r = - 0.294 P = 0.380	1	
Bacterial count on Nutrient Agar(Hi media) cfu/g	r = -0.148 P = 0.664	r = -0.375 P = 0.256	r = 0.030 P = 0.930	1

*** Correlation is significant at 0.05 level (2-tailed)**

****Correlation is significant at 0.01 level (2-tailed)**

5.1.2 Correlation coefficient of non-agricultural site soil samples

The correlation between the soil physicochemical properties and the bacterial count was determined using SPSS software. Table 5.4 indicated that there was a significant correlation between bacterial colony counts with soil physicochemical properties. Among these soil parameters of non-agriculture sites, only organic carbon content was correlated with the bacterial count at the significance level (0.01). Both pH and electrical conductivity were positively correlated with the bacterial count. Total bacterial count was positively correlated with organic carbon content (r = 0.984; P = 0.000), pH (r = 0.013; P= 0.973) and electrical conductivity (r = 0.793; P = 0.011) at (0.05) level of significance.

Table 5.4 Correlation coefficients between and within soil physiocochemical properties of non-agricultural site samples and bacterial growth (df =20)

Parameters	pH	Electrical conductivity (dS m-1)	Organic carbon content(g kg^{-1})	Bacterial count on Nutrient Agar(Hi media) CFU/g
pH	1			
Electrical conductivity (dS $m^{-1)}$	r = -0.062 P = 0.873	1		
Organic carbon content (g kg^{-1})	r =- 0.087 P = 0.823	r = - 0.767* P = 0.016	1	
Bacterial count on Nutrient Agar(Hi media) cfu/g	r = 0.013 P = 0.973	r = 0.793* P = 0.011	r=0.984** P = 0.000	1

**** Correlation is significant at 0.01 level (2-tailed)**

***Correlation is significant at 0.05 level (2-tailed)**

5.2 Bacterial isolation and identification

All bacterial strains from agricultural field as GRS , PAD3, BAM, BAN1, BAN2, PAD1, PAD2, ANUP, SUG1, MLK, SUG2 and from non-agricultural sites as MQG, SWY, SWV, PON1, VAG, CWT1, CWT2, DW1, PON2 were given strain codes based on their sources such as garden rhizosphere soil, paddy rhizosphere soil, bamboo rhizosphere soil, banana rhizosphere soil, cattle fodder farm soil, vegetable farm soil, mosquito breeding site, irrigational canal sediment, pond sediment respectively. The pure bacterial cultures were subjected to biochemical identification tests.

5.2.1 Biochemical identification of bacterial strains

Biochemical characteristics such as Gram staining, motility, catalase, oxidase, methyl red test, Indole test, Voges Proskaur test, nitrate reduction and gelatin liquefaction tests of all bacterial strains from agricultural sites are listed in Table 5.5. This result indicated that among 11 pure bacterial strains isolated, only four strains were gram negative bacteria and remaining strains were gram positive. This staining method used to distinguish bacterial strains based upon the morphological features in cell wall. All the isolated strains were motile and were effective root colonizers which move towards root exudates or nutritional ingredients. Catalase test showed positive in which all isolated bacterial strains produce catalase enzyme to break down the toxic by-products of oxygen metabolism. Catalase positive bacteria include strict aerobes and facultative anaerobes. Oxidase test show the detection of oxidase enzyme

which catalyzes the oxidation of cytochrome c as part of their respiratory chain. This test is particularly used to differentiate Pseudomonas and related species. Strains isolated from paddy rhizosphere PAD1, bamboo rhizosphere BAM, garden rhizosphere GRS, cattle fodder farm soil ANUP, vegetable farm MLK showed oxidase positive results. Nitrate reduction test is used to determine the production of enzyme nitrate reductase to reduce nitrate.

Table 5.5 Biochemical characterization of bacterial strains isolated from agricultural fields

S.No	Bacterial strains	Gram staining	Motility test	Catalase test	Oxidase test	Nitrate reduction test	Gelatin liquefaction	Indole test	Methyl test	VP test
1.	GRS	+	+	+	+	+	+	-	-	-
2.	PAD3	+	+	+	-	+	-	-	-	+
3.	BAM	-	+	+	+	+	+	-	-	-
4.	BAN1	+	+	+	-	+	-	-	-	+
5.	BAN2	+	+	+	-	+	-	-	-	+
6.	PAD1	-	+	+	+	+	+	-	-	-
7.	PAD2	+	+	+	-	+	-	-	-	+
8.	ANUP	-	+	+	+	+	+	-	-	-
9.	SUG1	+	+	+	-	+	-	-	-	+
10.	MLK	-	+	+	+	+	+	-	-	+
11.	SUG2	+	+	+	-	+	+	-	-	-

All bacterial strains isolated showed positive to nitrate reduction test. Gelatin liquefaction test is a presumptive test for the identification of *Staphylococcus, Enterococcus* and other Gram positive bacilli. Strains GRS, BAM, PAD1, ANUP, MLK, SUG2 showed positive to gelatin hydrolysis. Indole test and methyl red test were used to distinguish among members of the family Enterobacteriaceae which showed negative for all bacterial strains. Voges Proskauer (VP) test is used to differentiate two major types of facultative anaerobic enteric bacteria based on production of neutral products.

In this present study, the non-agricultural sites include pond sediment soil, sewage sediment and mosquito breeding site sediment soil were collected in sterile polythene bags. From each soil sample, pure bacterial cultures were isolated and provided with reference codes. Biochemical characteristics such as Gram staining, motility, catalase, oxidase, methyl red test, Indole test, Voges Proskaur test, nitrate reduction and gelatin liquefaction according to the standard procedures of bacterial isolates are shown in Table 5.6. This result indicated that among nine pure bacterial strains isolated, six strains were gram positive bacteria and remaining strains were gram negative. All strains were motile except PON1 strain isolated from pond sediment. The isolated strains were catalase positive which indicated aerobic organisms. Sewage sediment strains (SWY and SWV), Irrigation canal strain (CWT2) showed oxidase positive. In nitrate reduction test, only one strain isolated from pond sediment (PON1) showed negative result. All strains showed negative to Indole and methyl red test whereas MQG, VAG, CWT1, DW1, PON2 strains showed positive to Voges Proskauer test.

Table 5.6 Biochemical characterization of bacterial strains isolated from non- agricultural site samples

S.No	Bacterial strains	Gram staining	Motility test	Catalase test	Oxidase test	Nitrate reduction test	Gelatin liquefaction	Indole test	Methyl test	VP test
1.	MQG	+	+	+	-	+	-	-	-	+
2.	SWY	+	+	+	+	+	+	-	-	-
3.	SWV	+	+	+	+	+	+	-	-	-
4.	PON1	-	-	+	-	-	-	-	-	-
5.	VAG	+	+	+	-	+	-	-	-	+
6.	CWT1	-	+	+	-	+	-	-	-	+
7.	CWT2	-	+	+	+	+	+	-	-	-
8.	DW1	+	+	+	-	+	-	-	-	+
9.	PON2	+	+	+	-	+	-	-	-	+

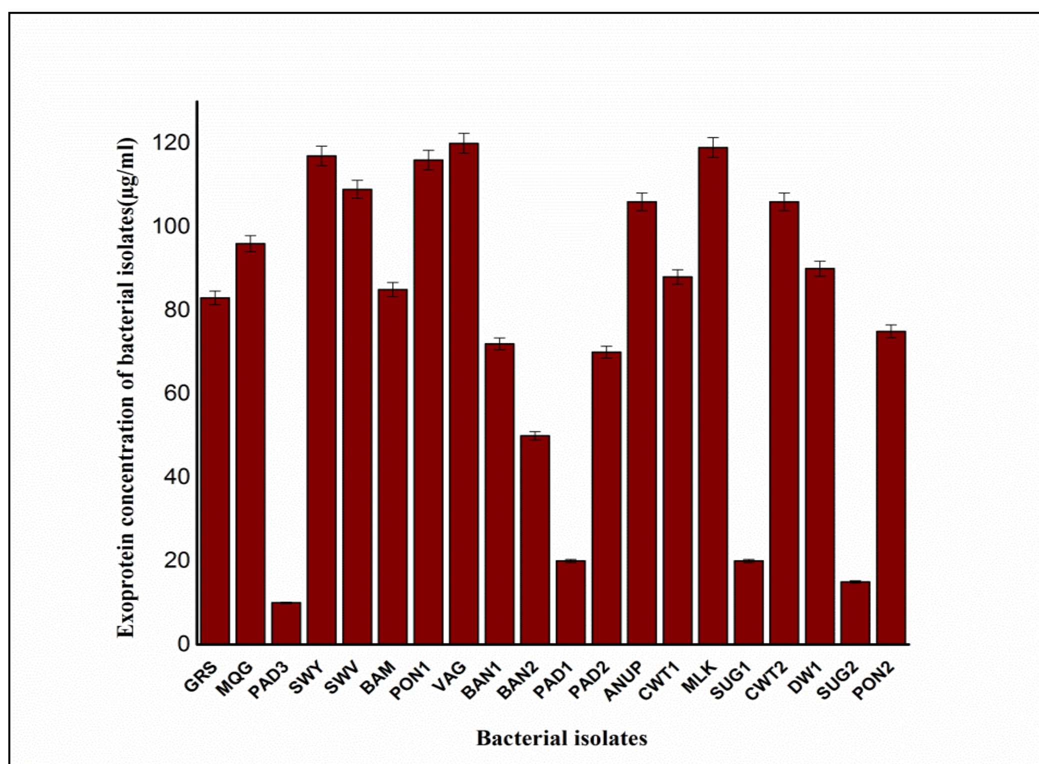
5.3 Toxigenicity of extracellular proteins isolated from bacterial strains

5.3.1 Partial purification of extracellular proteins

The extracellular proteins isolated from bacterial strains were characterized for protein estimation using Lowry's method. Results showed that the concentration of proteins ranges from 10 to 120 µg/ml. Bacterial strain from sediment soil produced a higher amount of extracellular proteins (120 $\pm$ 2.4) than other strains.

Fig 5.1 shows that sewage sediment (VAG) strain grown in Glucose Peptone broth secreted higher amount of exoproteins in culture filtrate followed by (MLK) strain from animal fodder farm, (SWY) strain from mosquito breeding site and (PON1) strain.

Fig 5.1 Extracellular concentration of bacterial strains from different sources

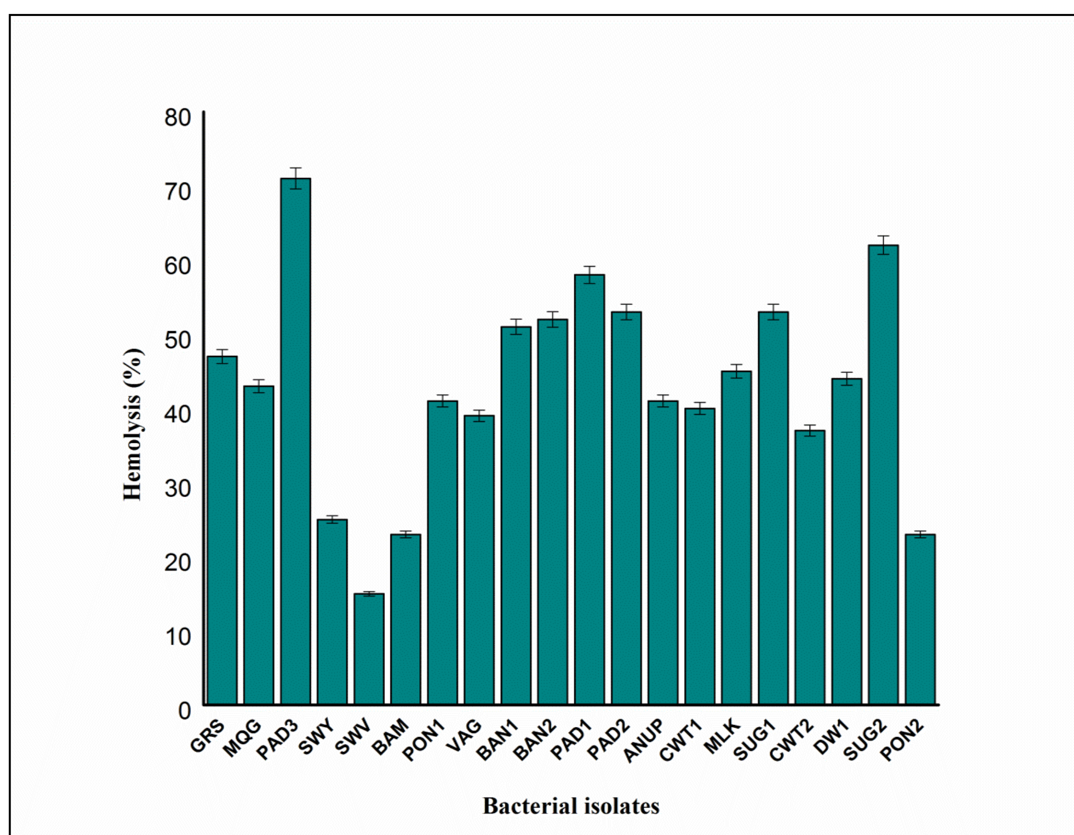


5.3.2 Evaluation of hemolytic activity of extracellular proteins

Hemolytic activity is a way to evaluate membrane disturbance which act as common feature present in entomopathogenic toxins. Therefore all bacterial strains posses mosquitocidal activity were screened for hemolytic ability of erythrocytes. All the extracellular proteins isolated from the bacterial strains were partially purified using 50% ammonium sulfate precipitation. These purified proteins were subjected to

hemolytic activity by using 1% sheep Red Blood Cells. The hemolytic percentage ranges from 15% to 71% which showed different pattern with respective of their sources (Fig 5.2). Bacterial strain (PAD3) isolated from paddy rhizosphere soil showed higher hemolytic activity (71±1.42) followed by (SUG2) strain from sugarcane rhizosphere and (PAD1) strain from paddy rhizosphere against S-RBC. Fig 5.2 indicated that the exoprotein concentration of 10 μg/ml excreted in the culture medium of PAD3 strain effectively lysis Sheep Red Blood cells.

Fig 5.2 Hemolytic activity of exoproteins extracted from bacterial strains against S-RBC



5.4 Screening of bacterial strains isolated from different sources

According to WHO Pesticides Evaluation Scheme (WHOPES), the guidelines were recommended for testing larvicides including bacterial larvicides and insect growth regulators for mosquitocidal activity. This helps to harmonize the testing procedures carried out in different laboratories and institutions to generate data for the registration and labeling of larvicides by national authorities. These tests also help to determine the dose-response line against susceptible vector species, the lethal concentration (LC) of larvicide for 50% and 90% mortality of mosquitoes, to establish a diagnostic concentration for monitoring susceptibility to mosquito larvicide in the field and to assess cross resistance with commonly used insecticides.

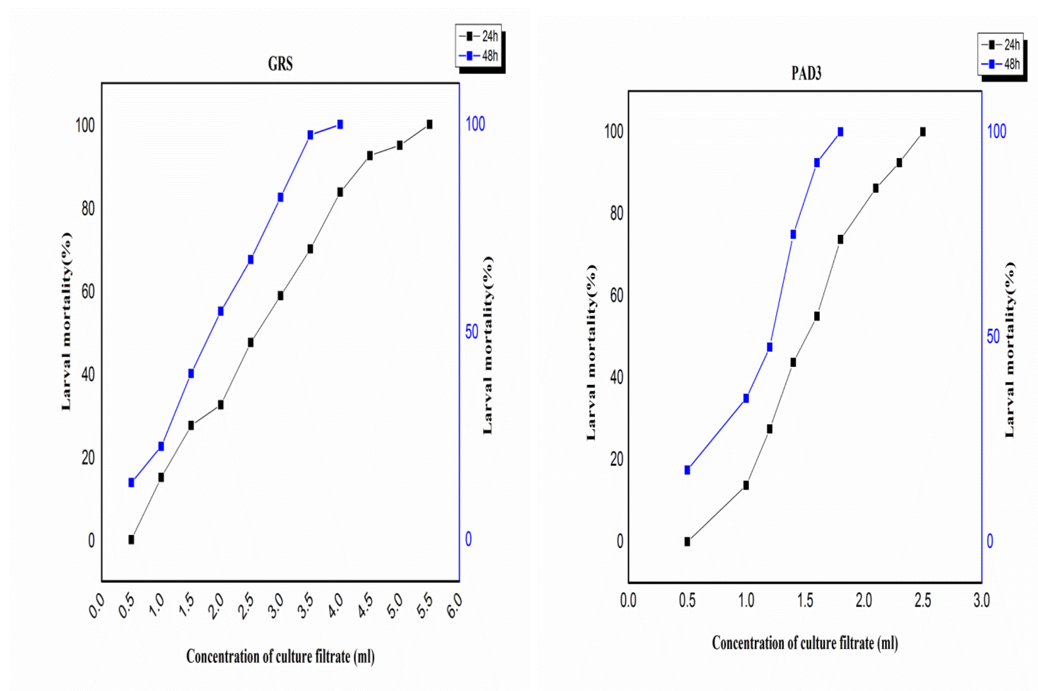
5.4.1 Biocontrol efficacy of bacterial strains against third instar larvae of mosquito, *Aedes aegypti*

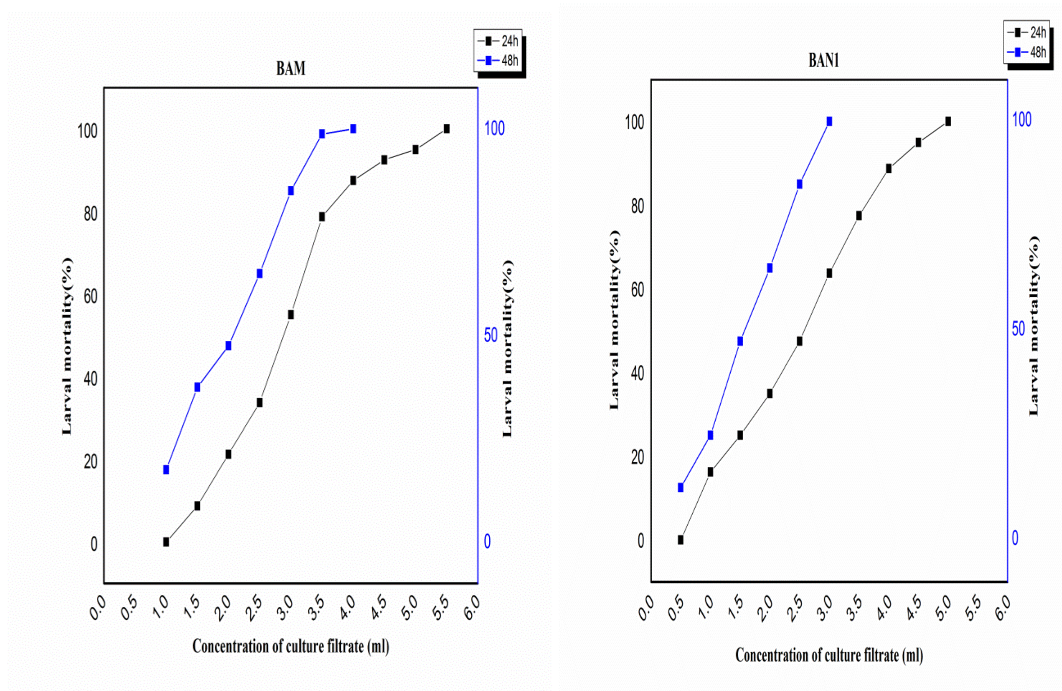
The exoproteins isolated from bacterial strains were tested against third instar larvae of Dengue fever vector, *Aedes aegypti.* The percentage mortality of *Aedes aegypti* larvae was corrected using Abbott's formula. The lethal concentration to cause 50% mortality (LC_{50}) and lethal concentration to cause 90% mortality (LC_{90}) were calculated using the computer software SPSS to determine the minimum concentration of tested bacterial isolate to cause maximum mortality of susceptible mosquitoes species.

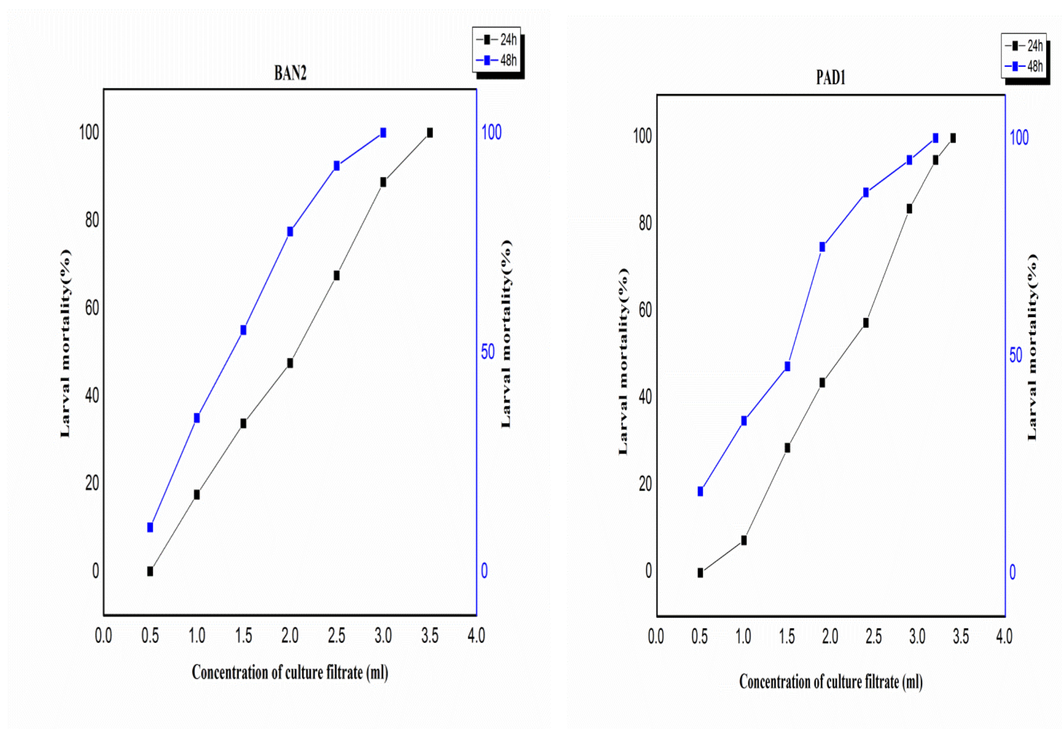
Fig 5.3 clearly showed the mortality rate of larvae for different concentrations of extracellular proteins from bacterial strains of the agricultural field after 24 hrs and

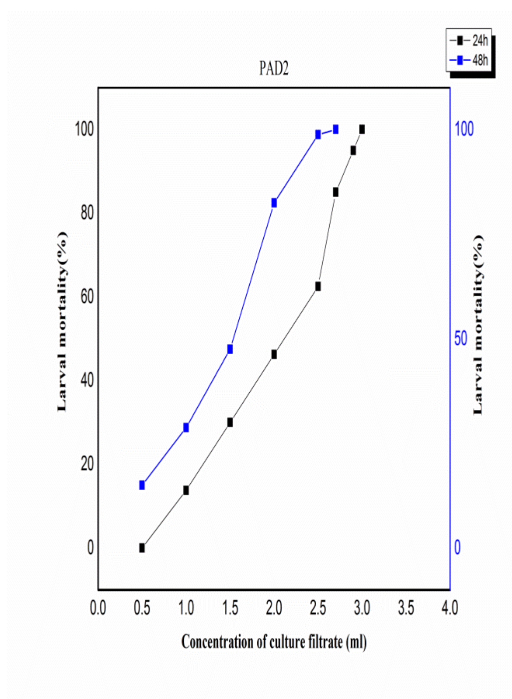
48 hrs treatment.The larvicidal activity was recorded highest in bacterial strains from the paddy field and sugarcane field (PAD3, SUG2 and PAD1). After 48 hrs treatment, the larvicidal activity of three bacterial strains were recorded as 50% in 12 µg/ml and 100% in18 µg/ml; 50% in 22.5 µg/ml and 100% in 39 µg/ml; 50% in 38 µg/ml and 100% in 64 µg/ml respectively. In this present study, the mortality percentage of *Aedes aegypti* larvae by three bacterial strains (PAD3, PAD1, and SUG2) increased with an increase in the exposure time.

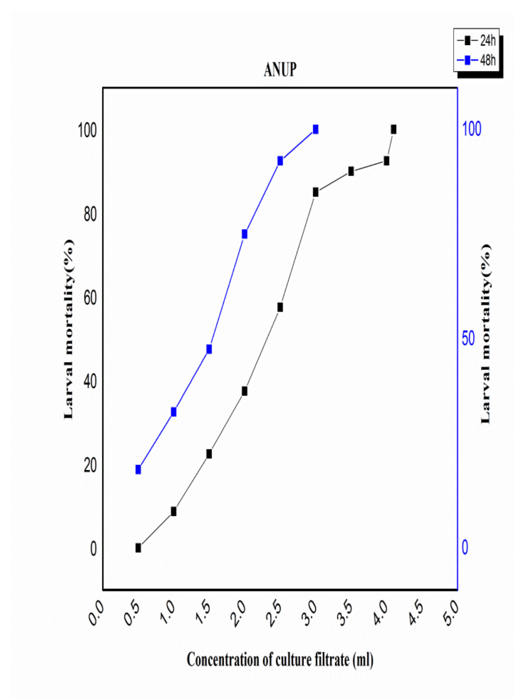
Fig 5.3 Larvicidal activity of bacterial strains from agricultural field against third instar larvae of mosquito vector, *Aedes aegypti* during 24 hrs and 48 hrs of treatment.

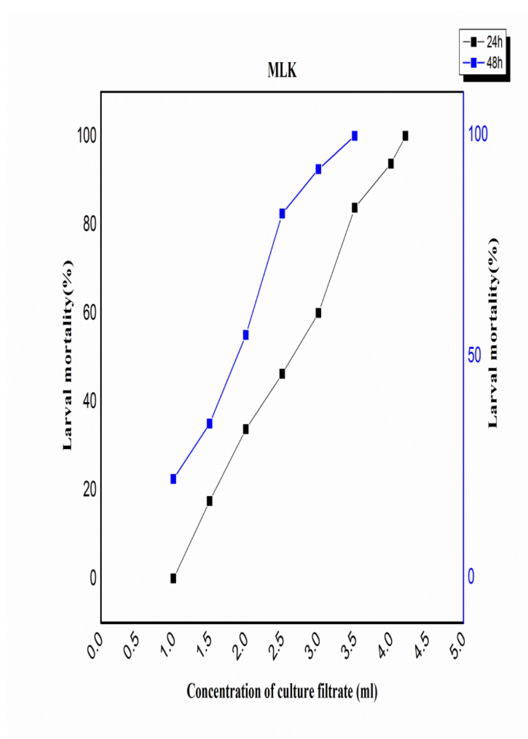


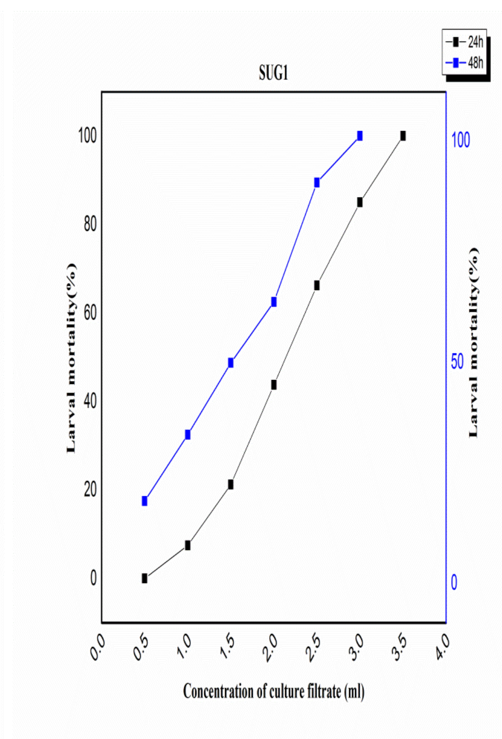

BAM
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)
BAN1
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)


BAN2
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)
PAD1
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)


PAD2
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)


ANUP
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)


MLK
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)


SUG1
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)

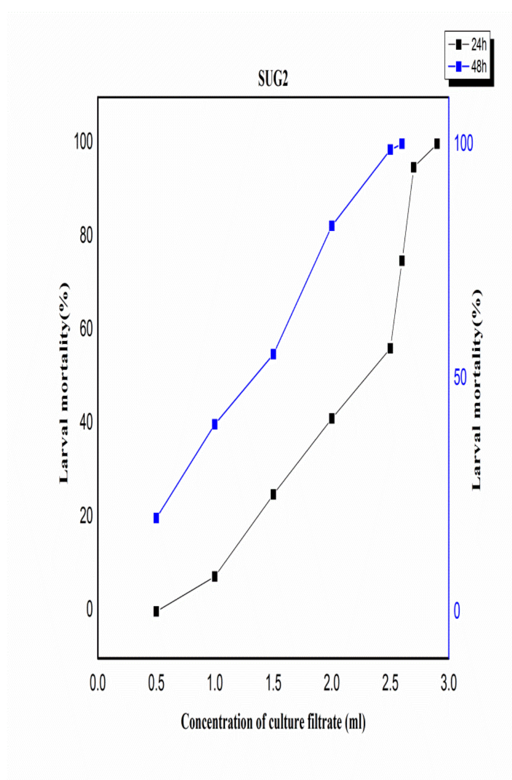


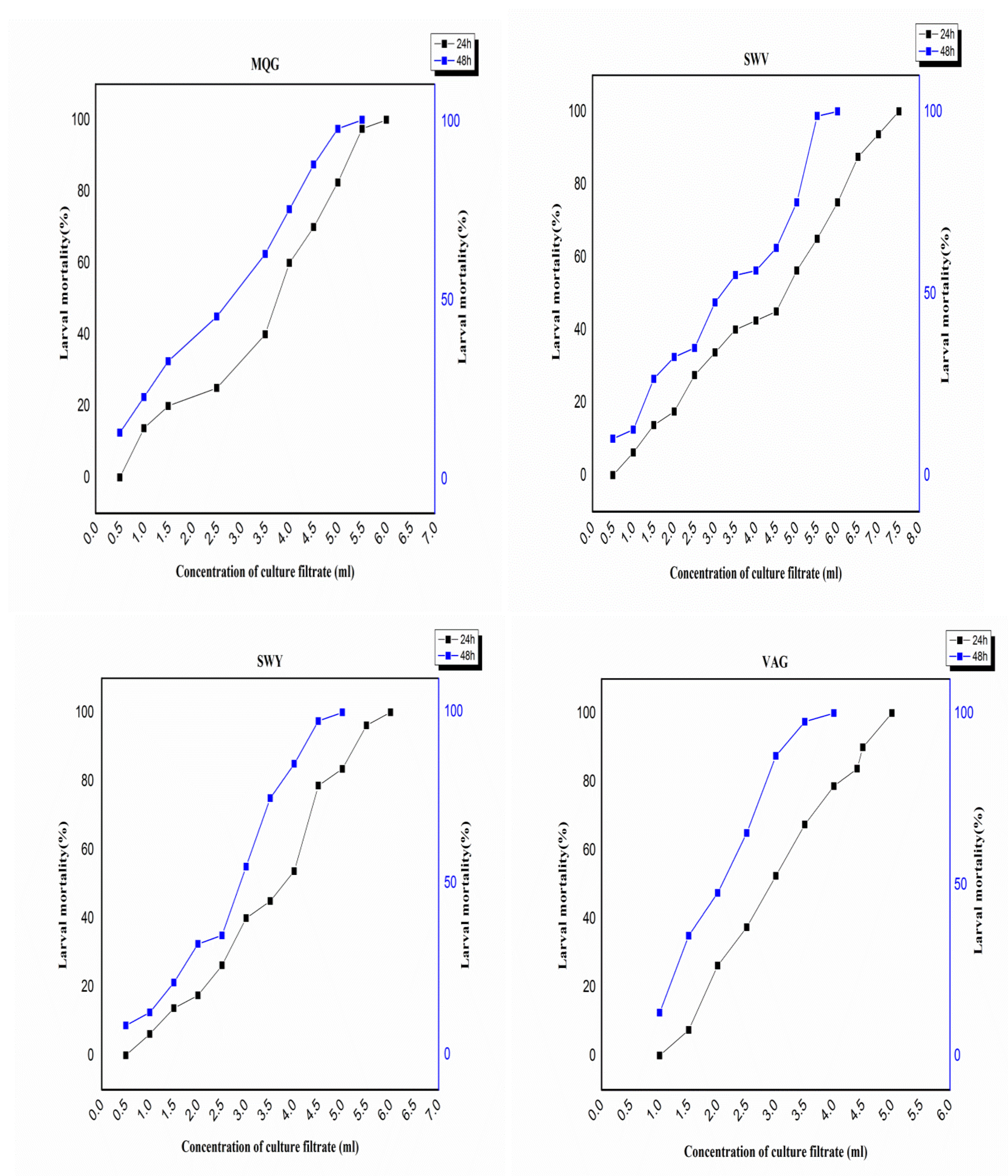
Table 5.7 indicated that LC_{50} value and LC_{90} of highly effective bacterial strains (PAD3, SUG2 and PAD1) isolated from paddy field and sugarcane field were 15.193 µg/ml and 21.277 µg/ml, 30.817 µg/ml and 43.077 µg/ml, 41.354 µg/ml, and 60.067 µg/ml. This reported that bacterial strain isolated from paddy rhizosphere showed highest larvicidal activity against *Aedes agypti* larvae.

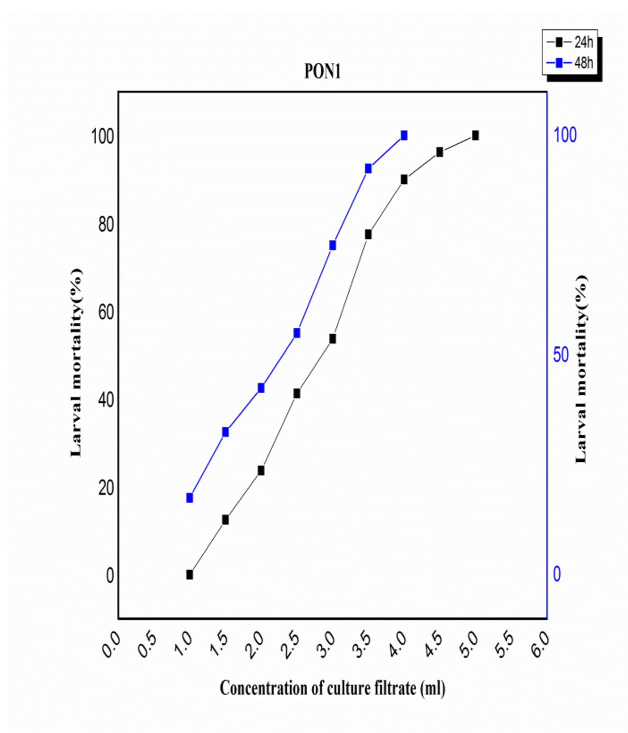
Table 5.7 Lethal Concentration of exoproteins of bacterial strains isolated from agricultural field against third instar larvae of *Aedes aegypti.*

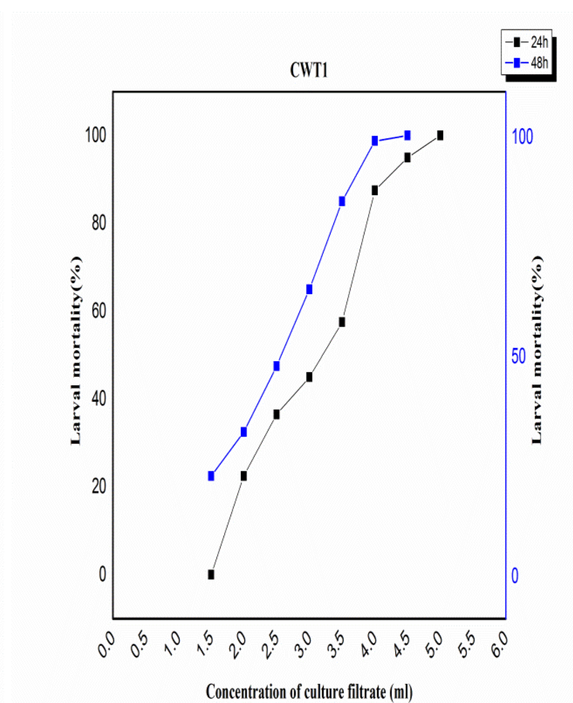
S.No.	Bacterial strains	LC_{50}(µg/ml) 95% FL	LC_{90}(µg/ml) 95% FL
1.	GRS	217.646 (193.401-240.895)	361.338 (329.017-407.640)
2.	**PAD3**	**15.193 (14.018-16.306)**	**21.277 (19.763-588.297)**
3.	BAM	264.073 (225.073-266.364)	356.471(329.789-394.545)
4.	BAN1	179.613 (159.976-198.689)	290.915 (264.544-329.306)
5.	BAN2	98.542 (87.473-109.599)	150.856 (136.060-174.172)
6.	PAD1	41.354 (37.415-45.141)	60.067 (55.181-67.276)
7.	PAD2	137.482 (124.085-149.591)	200.222 (184.847-222.760)
8.	ANUP	241.512 (217.009-265.070)	364.586 (334.111-408.251)
9.	MLK	304.416 (276.565-331.169)	442.482 (406.908-495.876)
10.	SUG1	42.609 (38.532-46.766)	61.111 (55.692-69.577)
11.	SUG2	30.817 (28.197-33.171)	43.077 (40.021-47.695)

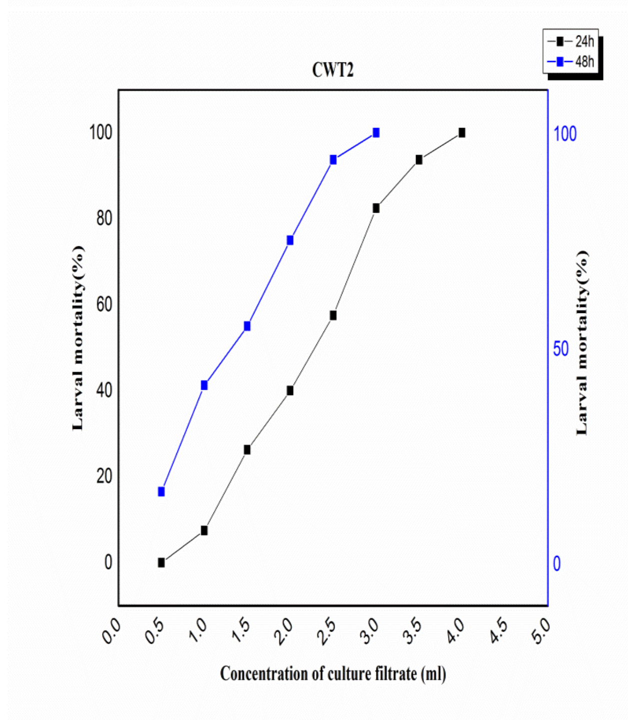
Fig 5.4 showed the mortality rate of larvae for different concentrations of extracellular proteins from bacterial strains of non-agricultural sites after 24 hrs and 48 hrs treatment. The LC_{50} and LC_{90} values of bacterial strain from pond sediment (PON2) showed as 232.097 (214.583-249.266) and 318.861 (295.876-353.735) µg/ml, respectively, which showed higher mortality when compared with other non-agricultural sites in Table 5.8. In this present study, the mortality percentage of *Aedes aegypti* larvae by bacterial strain increased with an increase in the exposure time after 48 hrs treatment.

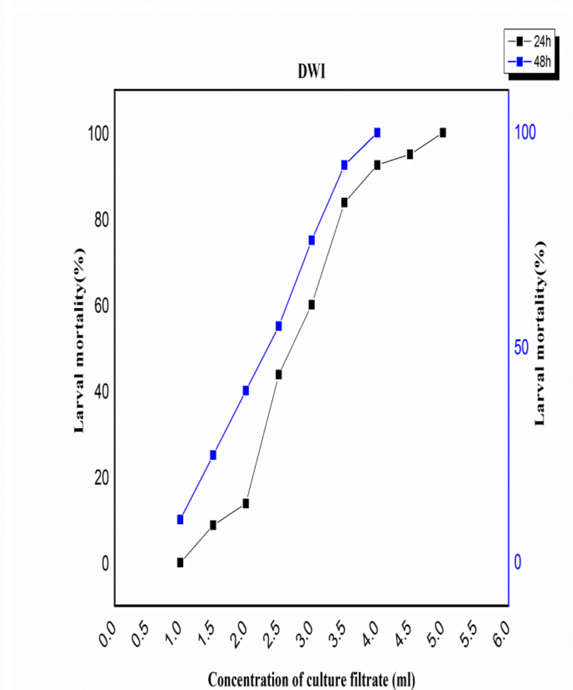
Fig 5.4 Larvicidal activity of bacterial strains from non-agricultural field against third instar larvae of mosquito vector, *Aedes aegypti* during 24 hrs and 48 hrs of treatment




PON1
24h
48h
Larval mortality(%)
Larval mortality(%)
Concentration of culture filtrate (ml)


CWT1
24h
48h
Larval mortality(%)
Larval mortality(%)
Concentration of culture filtrate (ml)


CWT2
24h
48h
Larval mortality(%)
Larval mortality(%)
Concentration of culture filtrate (ml)


DWI
24h
48h
Larval mortality(%)
Larval mortality(%)
Concentration of culture filtrate (ml)

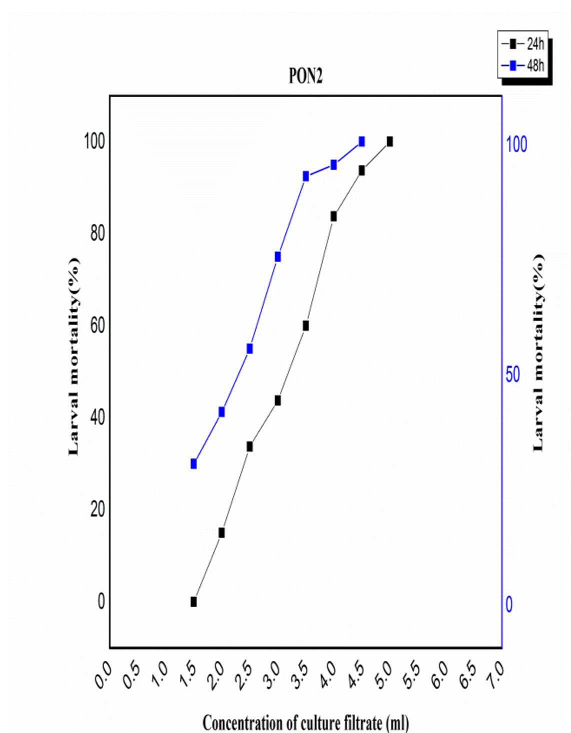


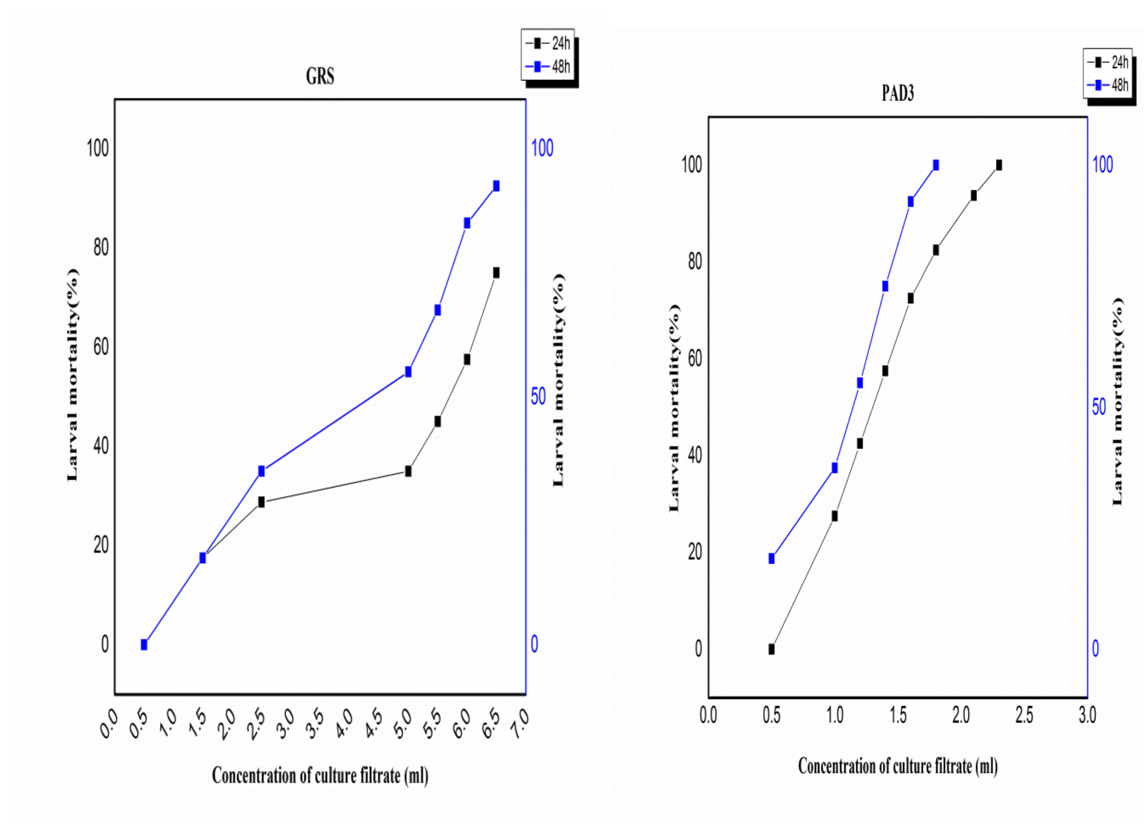
Table 5.8 Lethal concentration of exoproteins of bacterial strains isolated from non-agricultural sites against third instar larvae of *Aedes aegypti*

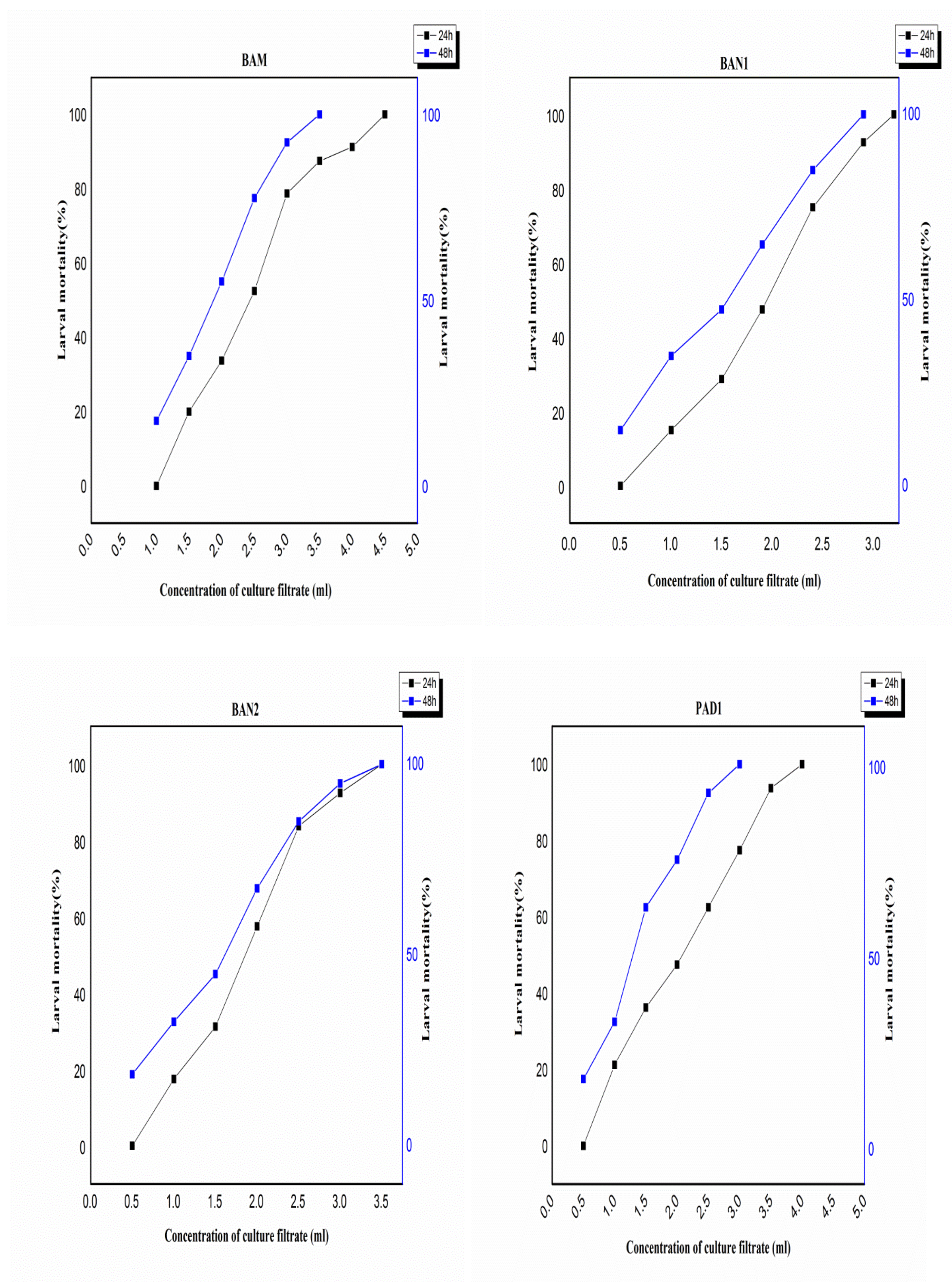
S.No.	Bacterial strains	LC_{50}(µg/ml) 95% FL	LC_{90}(µg/ml) 95% FL
1.	MQG	326.040 (291.709-359.379)	522.401 (476.588-588.297)
2.	SWY	402.049 (368.462-436.488)	616.840 (567.408-688.893)
3.	SWV	461.870 (422.507-502.349)	761.207 (698.198-849.714)
4.	PON1	321.387 (293.856-348.353)	462.362 (426.521-515.003)
5.	VAG	358.278 (327.307-387.470)	531.644 (492.141-588.325)
6.	CWT1	265.546 (244.404-297.282)	371.339 (343.567-413.784)
7.	CWT2	234.283 (211.138-257.384)	345.960 (315.557-391.777)
8.	DW1	249.270 (229.175-268.936)	347.103 (321.685-384.093)
9.	**PON2**	**232.097 (214.583-249.266)**	**318.861 (295.876-353.735)**

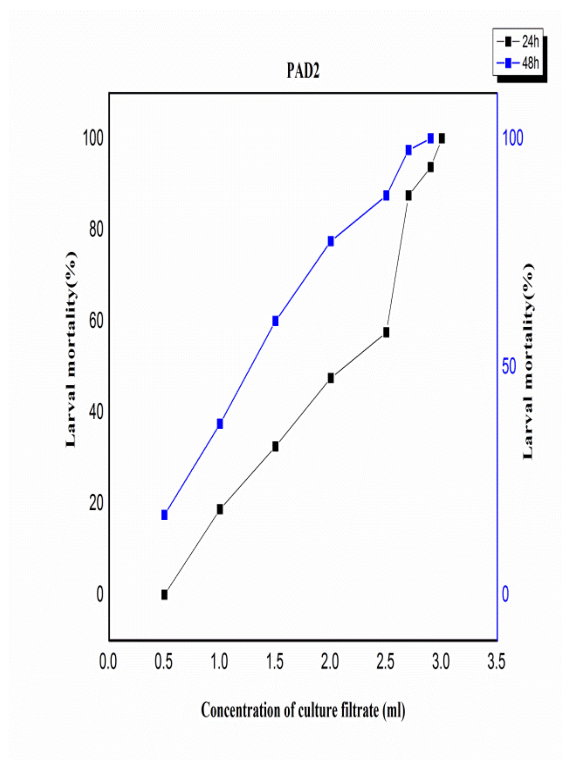
5.4.2 Biocontrol efficacy of bacterial strains against third instar larvae of mosquito, *Culex quinquefasciatus*

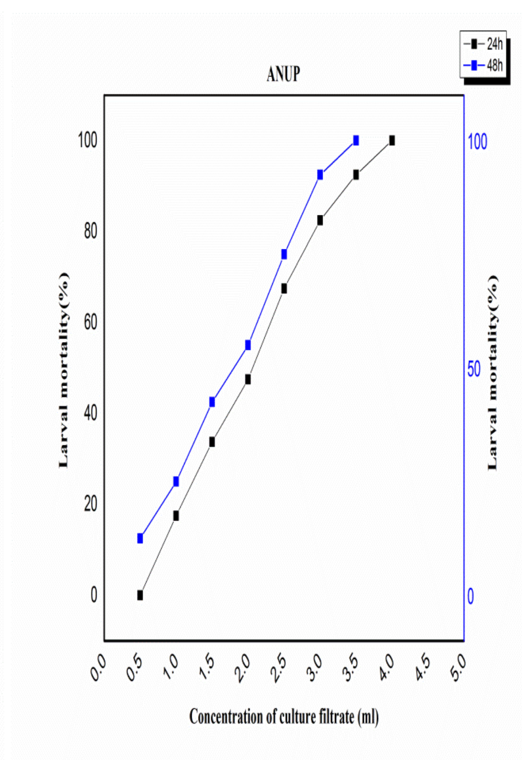
Similarly, the exoproteins isolated from bacterial strains were tested against third instar larvae of Filariasis vector, *Culex quinquefasciatus.* Fig 5.5 showed the mortality rate of larvae for all the tested bacterial culture filtrate after 24 hrs and 48 hrs treatment.

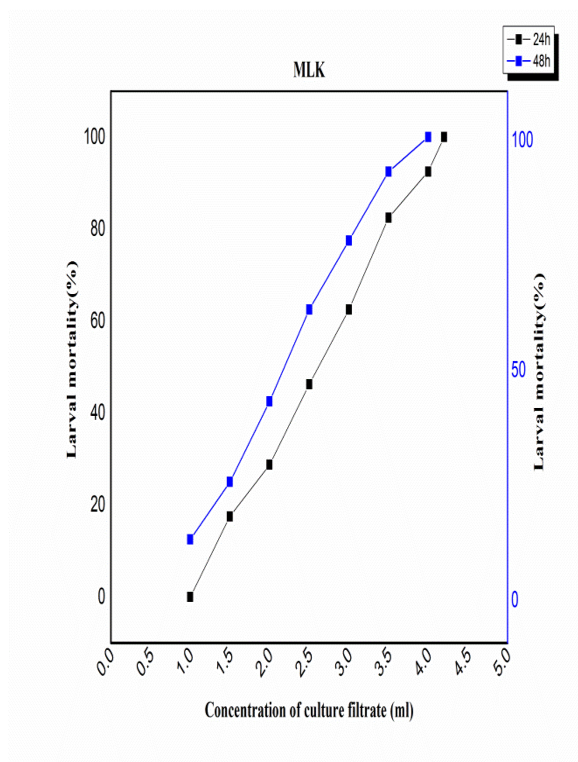
Fig 5.5 Larvicidal activity of bacterial strains from agricultural field against third instar larvae of mosquito vector, *Culex quinquefasciatus* during 24 hrs and 48 hrs of treatment.

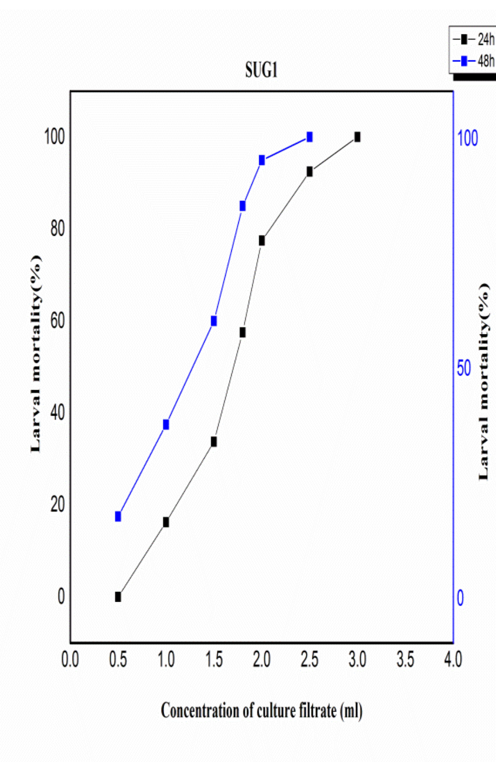


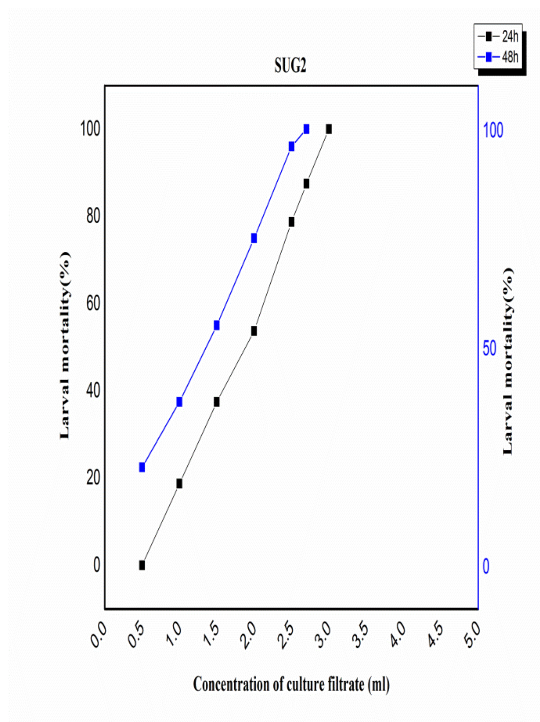

BAM
BAN1
BAN2
PAD1
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)


PAD2
24h
48h
Larval mortality(%)
Larval mortality(%)
Concentration of culture filtrate (ml)


ANUP
24h
48h
Larval mortality(%)
Larval mortality(%)
Concentration of culture filtrate (ml)


MLK
24h
48h
Larval mortality(%)
Larval mortality(%)
Concentration of culture filtrate (ml)


SUG1
24h
48h
Larval mortality(%)
Larval mortality(%)
Concentration of culture filtrate (ml)



During 24 hrs treatment, the larvicidal activity was recorded highest in bacterial strains from the paddy field and sugarcane field (PAD3, SUG2, and PAD1). After 48 hrs treatment, the larvicidal activity of bacterial strains were recorded as 50% in 42.5 µg/ml and 100% in 82.5 µg/ml; 50% in 22.5 µg/ml and 100% in 40.5 µg/ml; 50% in 30 µg/ml and 100% in 60 µg/ml. In this present study, the mortality percentage of *Culex quinquefasciatus* larvae by three bacterial strains (PAD3, PAD1 and SUG2) increased with an increase in the exposure time.

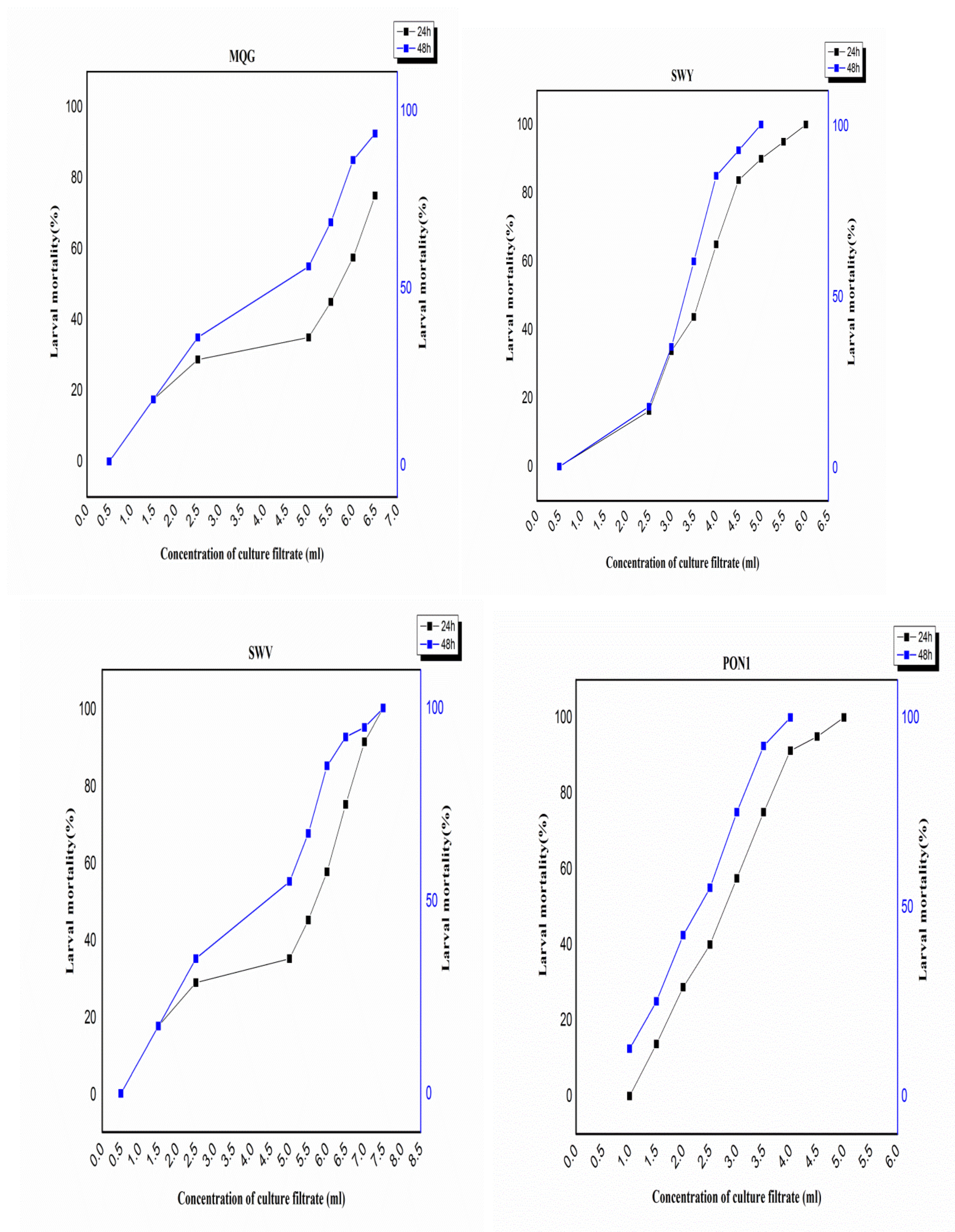
The Probit Regression analysis was carried out to calculate LC_{50} value and LC_{90} value of bacterial strains tested in the bioassay experiment. Table 5.9 indicated that the LC_{50} value and LC_{90} of bacterial strains (PAD3, SUG2 and PAD1) isolated from paddy field and sugarcane field were 13.268 µg/ml and 18.975 µg/ml, 27.221 µg/ml and 40.907 µg/ml, 40.951 µg/ml, and 66.304 µg/ml respectively.

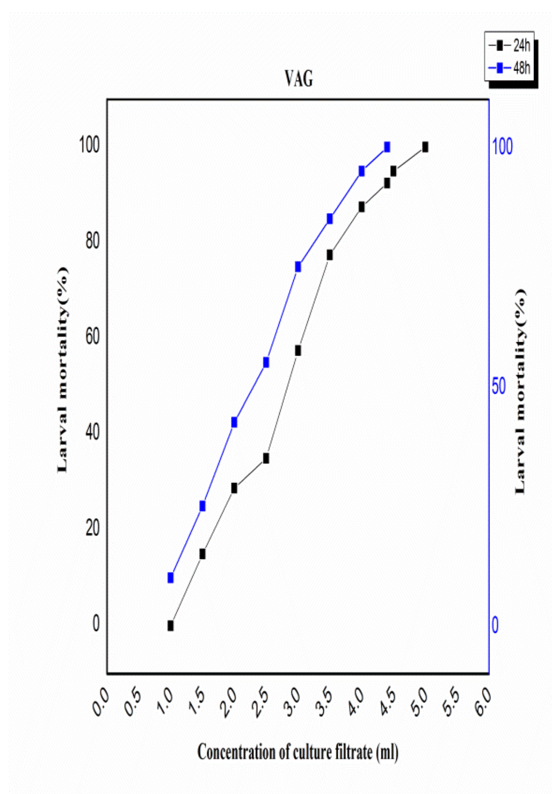
Table 5.9 Lethal concentration of exoproteins of bacterial strains isolated from agricultural field against third instar larvae of *Culex quinquefasciatus*

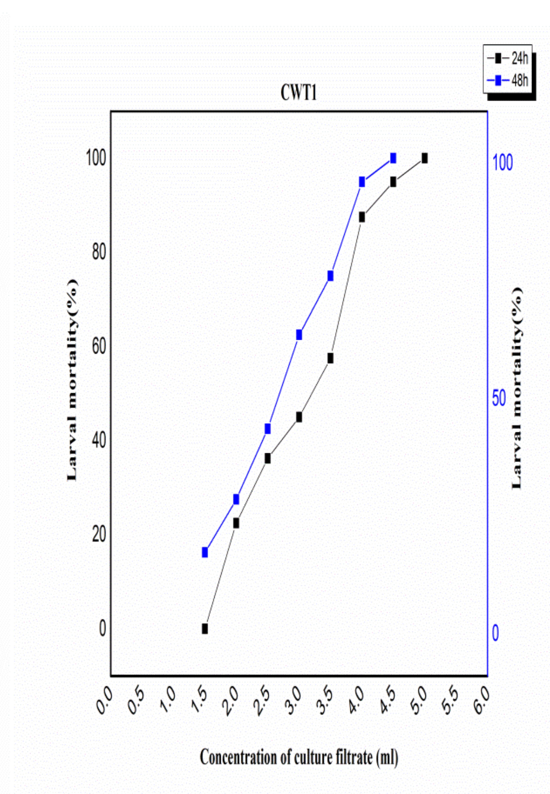
S.No.	Bacterial isolates	LC_{50}(µg/ml) 95% FL	LC_{90}(µg/ml) 95% FL
1.	GRS	138.248(120.136-155.379)	217.362(195.248-251.946)
2.	**PAD3**	**13.268(12.032-14.359)**	**18.975(17.528-21.243)**
3.	BAM	206.804(186.390-226.114)	304.853(279.491-343.027)
4.	BAN1	135.602(121.814-149.231)	197.602(179.968-224.810)
5.	BAN2	91.875(81.559-101.973)	137.358(124.388-157.389)
6.	PAD1	40.951(35.942-45.782)	66.304(59.709-76.444)
7.	PAD2	135.278(121.224-147.985)	202.800(186.333-227.253)
8.	ANUP	216.355(190.918-240.911)	342.189(309.324-392.056)
9.	MLK	307.546(279.850-334.237)	444.980(409.536-498.039)
10.	SUG1	33.232(30.072-36.253)	46.981(43.005-53.445)
11.	SUG2	27.221(24.189-30.038)	40.907(37.275-46.449)

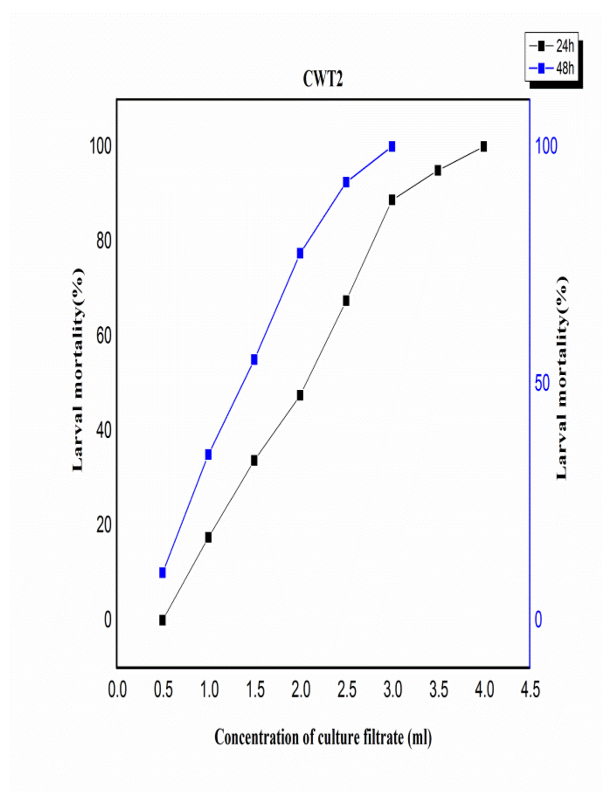
During 24 hrs treatment, the larvicidal activity was recorded highest in bacterial strain from mosquito breeding site (MQG) compared to other strains from the non-agricultural field. In this present study, the mortality percentage of *Culex quinquefasciatus* larvae by bacterial strain (MQG) increased with an increase in the exposure time in Fig 5.6. The Probit Regression analysis was carried out to calculate the LC_{50} value and LC_{90} value of bacterial strains tested in the bioassay experiment. Table 5.10 indicated that the LC_{50} value and LC_{90} of bacterial strain (MQG) isolated from mosquito breeding site was 205.595 (µg/ml and 346.877 (µg/ml) respectively.

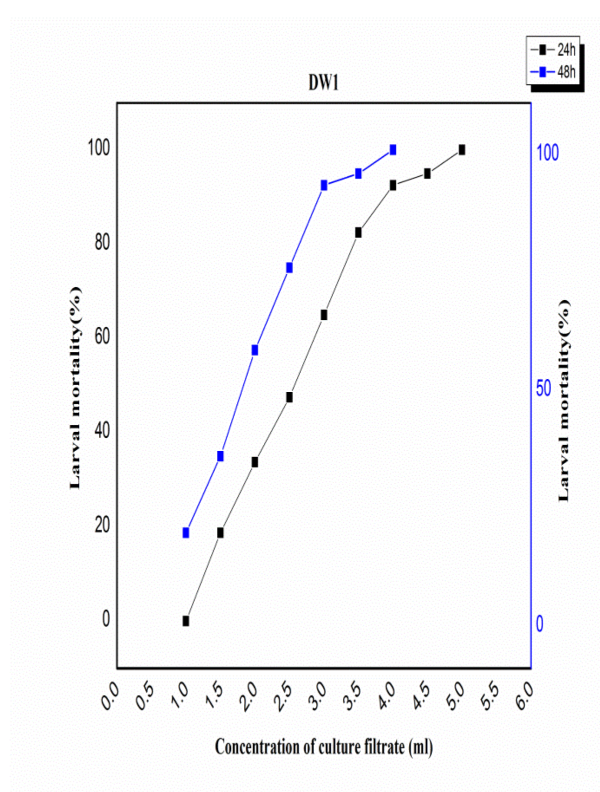
Fig 5.6 Larvicidal activity of bacterial strains from non-agricultural field against third instar larvae of mosquito vector, *Culex quinquefasciatus* during 24 hrs and 48 hrs of treatment.




VAG
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)


CWT1
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)


CWT2
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)


DW1
24h
48h
Larval mortality(%)
Concentration of culture filtrate (ml)

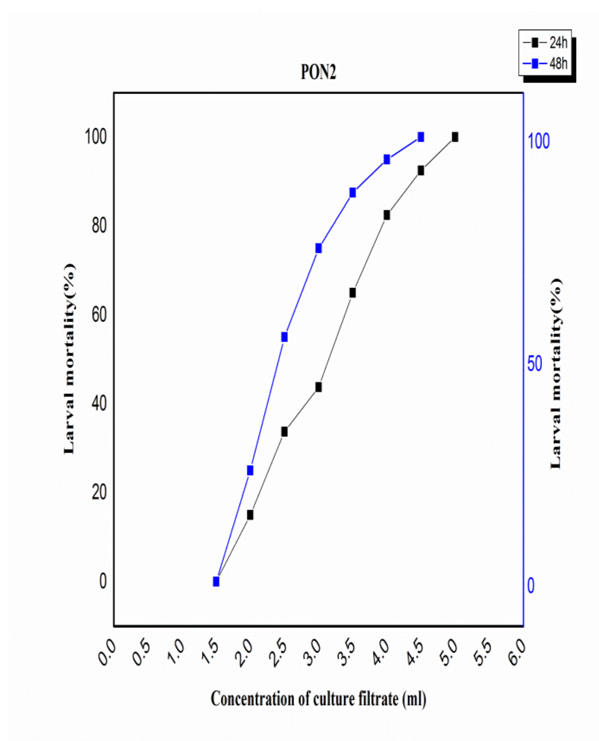


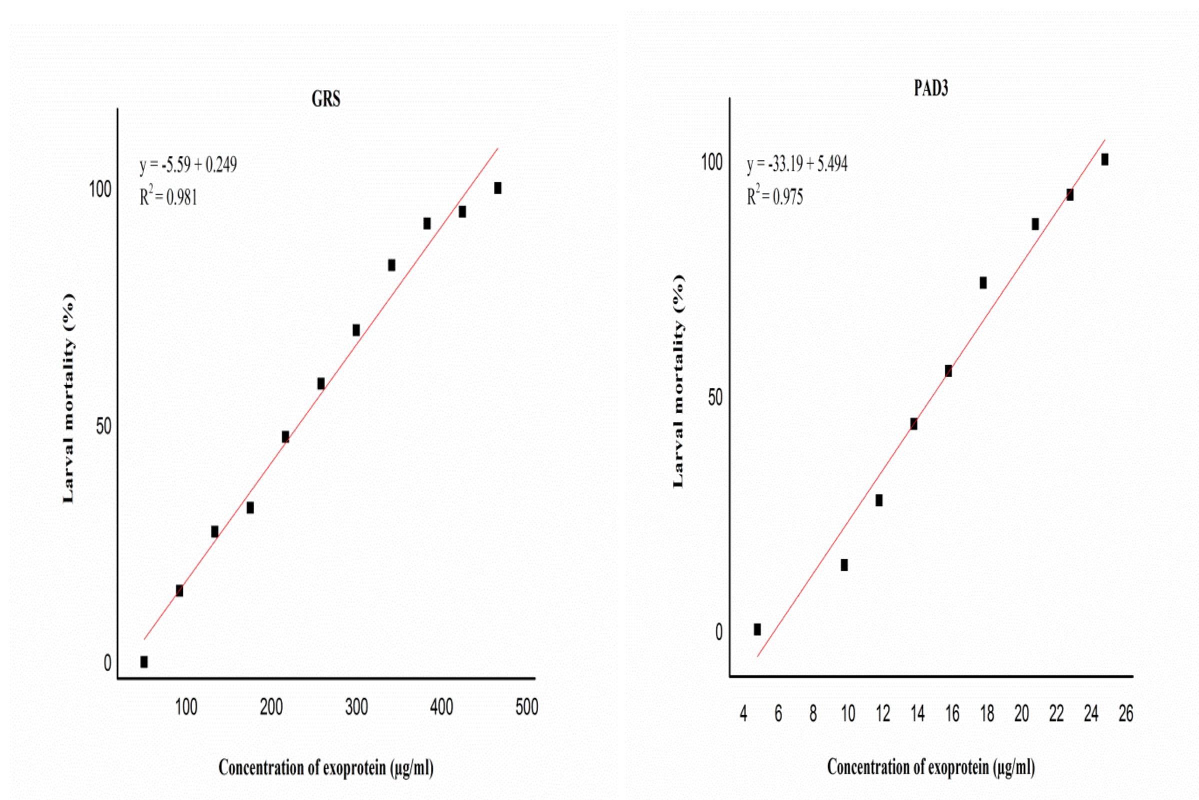
Table 5.10 Lethal concentration of bacterial strains isolated from non-agricultural sites against third instar larvae of *Culex quinquefasciatus*

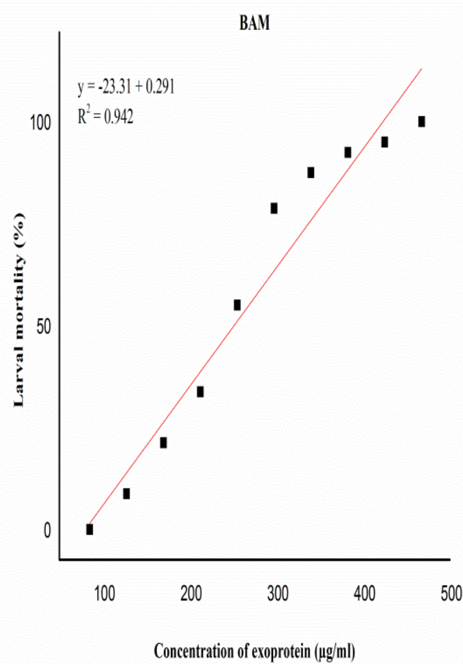
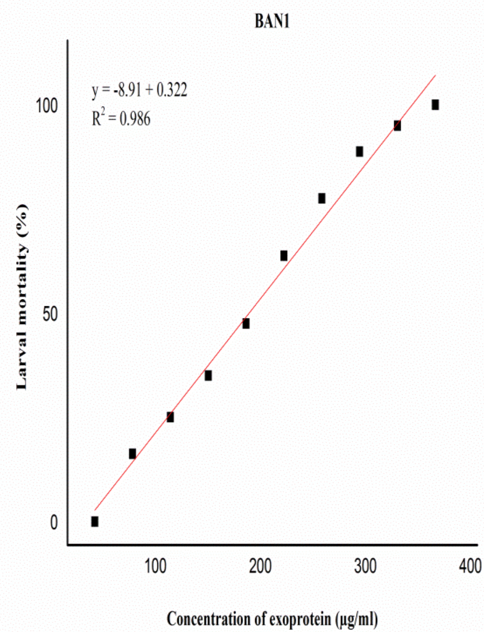
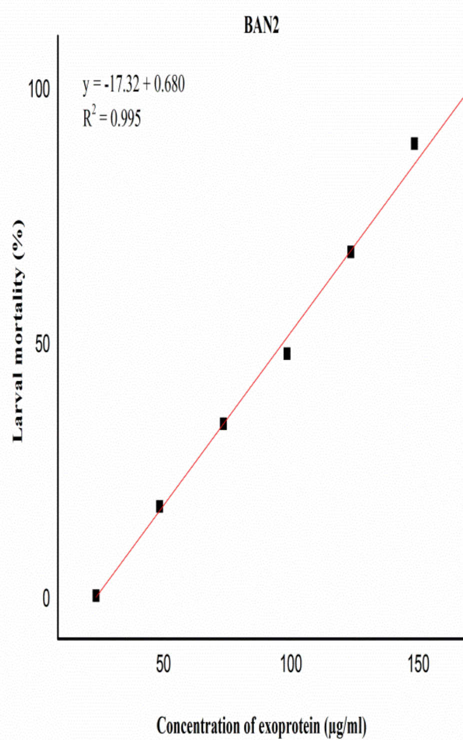
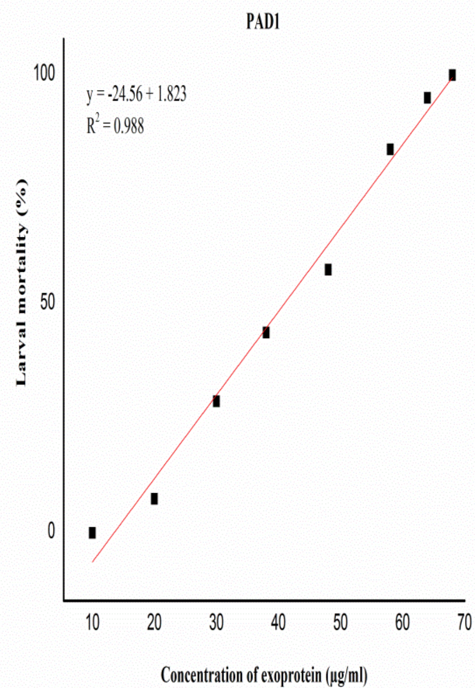
S.No.	Bacterial strains	LC_{50}(µg/ml) 95% FL	LC_{90}(µg/ml) 95% FL
1.	**MQG**	**205.595(174.403-235.903)**	**346.877(308.009-405.504)**
2.	SWY	416.556(381.955-446.619)	577.002(537.814-637.133)
3.	SWV	525.626(405.194-633.149)	871.042(738.186-1177.809)
4.	PON1	317.710(289.321-345.289)	464.653(427.755-518.994)
5.	VAG	332.760(302.943-360.591)	490.662(454.574-541.433)
6.	CWT1	265.656(244.433-286.233)	371.355(343.600-413.768)
7.	CWT2	211.560(187.234-235.119)	328.729(297.677-375.620)
8.	DW1	231.359(208.552-252.892)	346.940(318.401-389.050)
9.	PON2	231.359(213.675-248.600)	318.960(295.892-353.924)

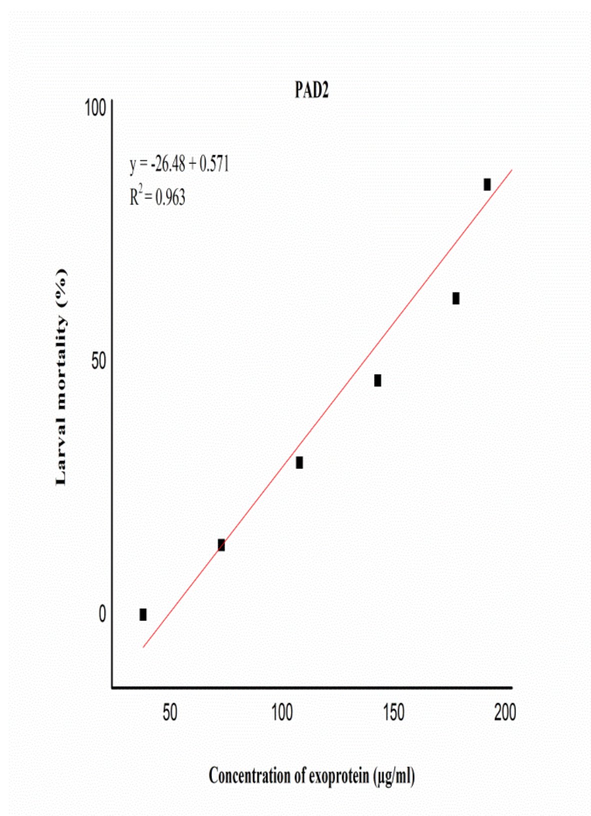
5.4.3 Regression analysis

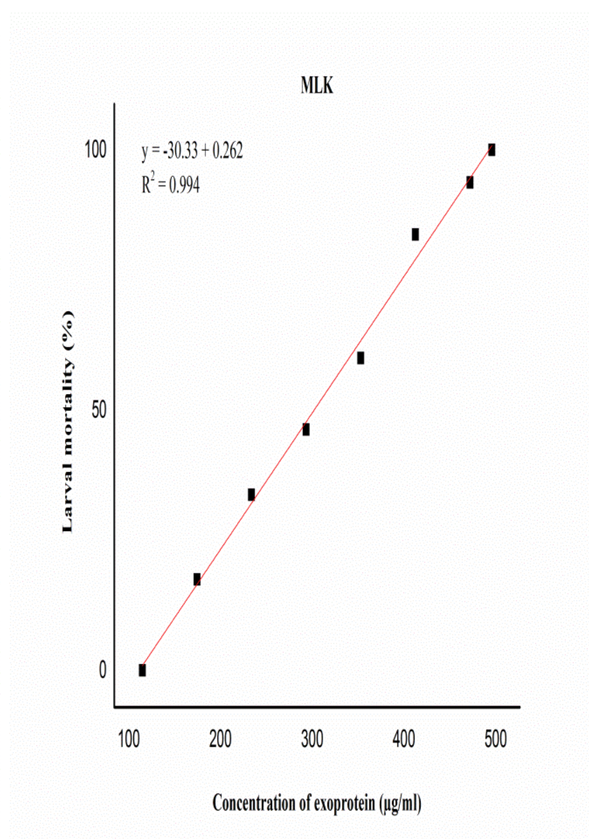
The experimental data was analyzed using regression equation (Y= mortality; X= concentration) and corresponding regression coefficient values using the SPSS software. The results of regression analysis reported that the mosquito larval mortality rate (Y) of *Aedes aegypti* was significantly positively correlated with the concentration of bacterial exoproteins extracted from agricultural field soil samples with respect to exposure period ($p<0.05$) and the regression coefficient values were calculated for PAD3, SUG2, PAD1 (R^2 =0.975), (R^2 =0.921) and (R^2 =0.988) respectively (Fig 5.7).

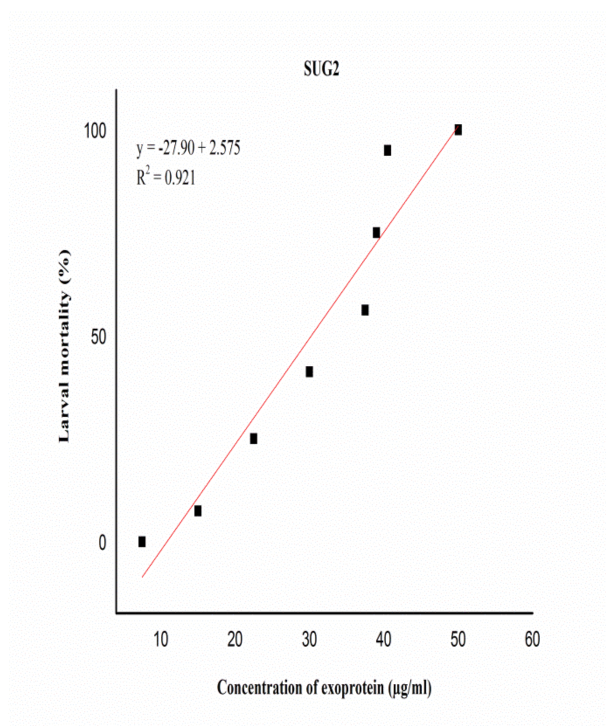
Fig 5.7 Evaluation of biocontrol efficacy of bacterial strains from agricultural field samples against third instar larvae of *Aedes aegypti*.

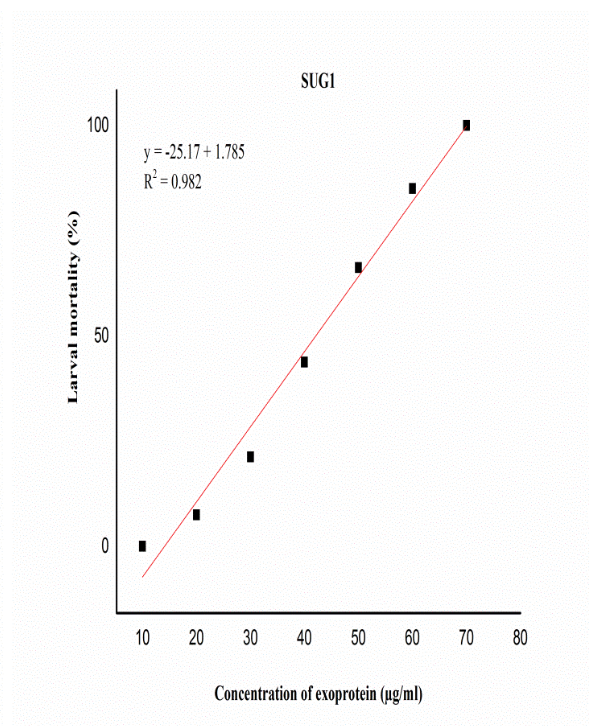



BAM
y = -23.31 + 0.291
R2 = 0.942
Larval mortality (%)
100
50
0
100
200
300
400
500
Concentration of exoprotein (µg/ml)

BAN1
y = -8.91 + 0.322
R2 = 0.986
Larval mortality (%)
100
50
0
100
200
300
400
Concentration of exoprotein (µg/ml)

BAN2
y = -17.32 + 0.680
R2 = 0.995
Larval mortality (%)
100
50
0
50
100
150
Concentration of exoprotein (µg/ml)

PAD1
y = -24.56 + 1.823
R2 = 0.988
Larval mortality (%)
100
50
0
10
20
30
40
50
60
70
Concentration of exoprotein (µg/ml)


PAD2
y = -26.48 + 0.571
R2 = 0.963
Larval mortality (%)
Concentration of exoprotein (μg/ml)

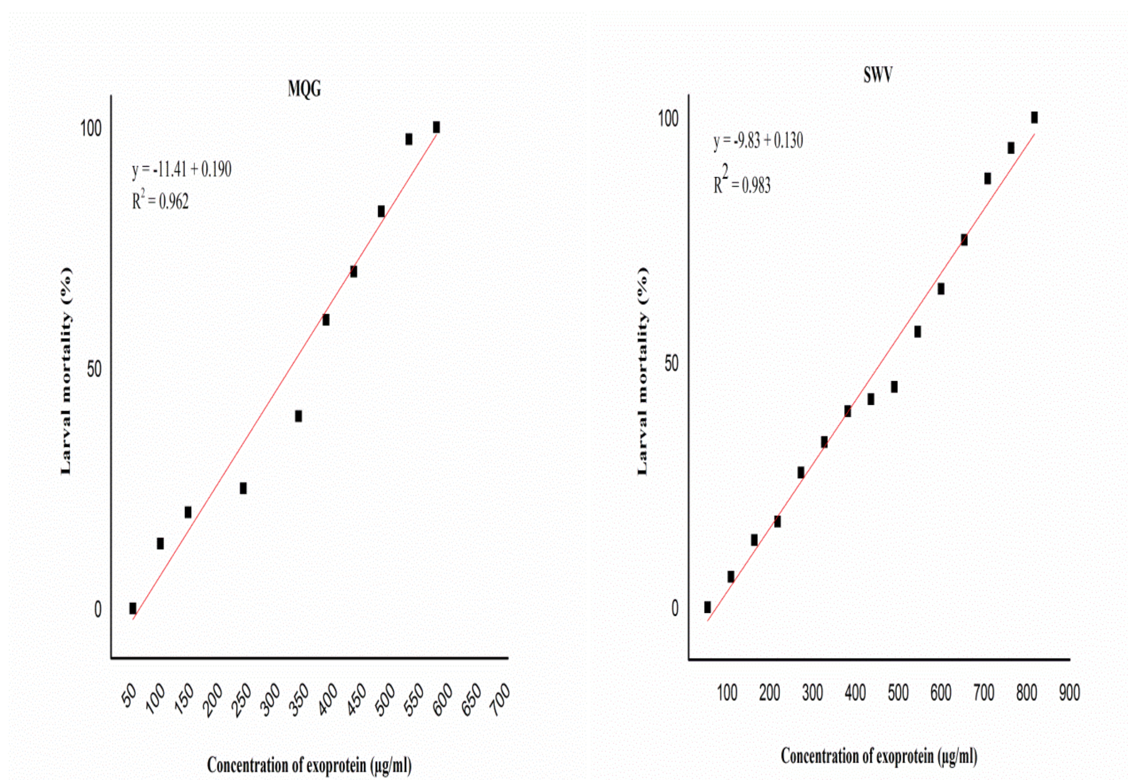

MLK
y = -30.33 + 0.262
R2 = 0.994
Larval mortality (%)
Concentration of exoprotein (μg/ml)

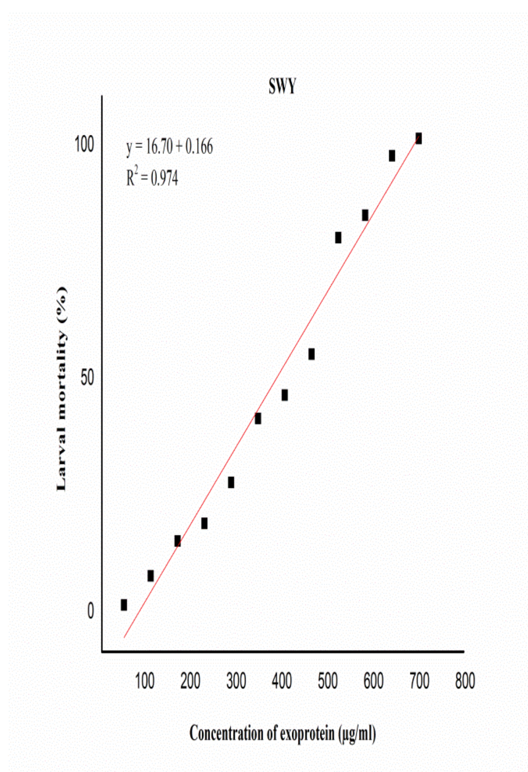

SUG2
y = -27.90 + 2.575
R2 = 0.921
Larval mortality (%)
Concentration of exoprotein (μg/ml)

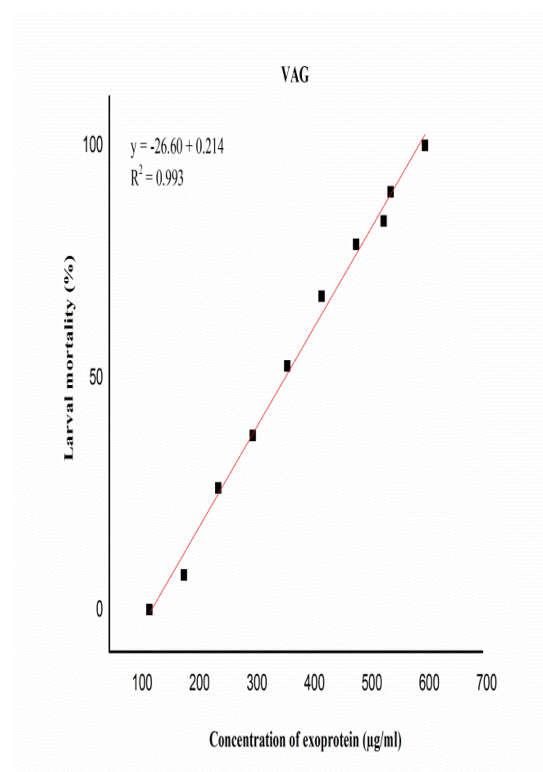

SUG1
y = -25.17 + 1.785
R2 = 0.982
Larval mortality (%)
Concentration of exoprotein (μg/ml)

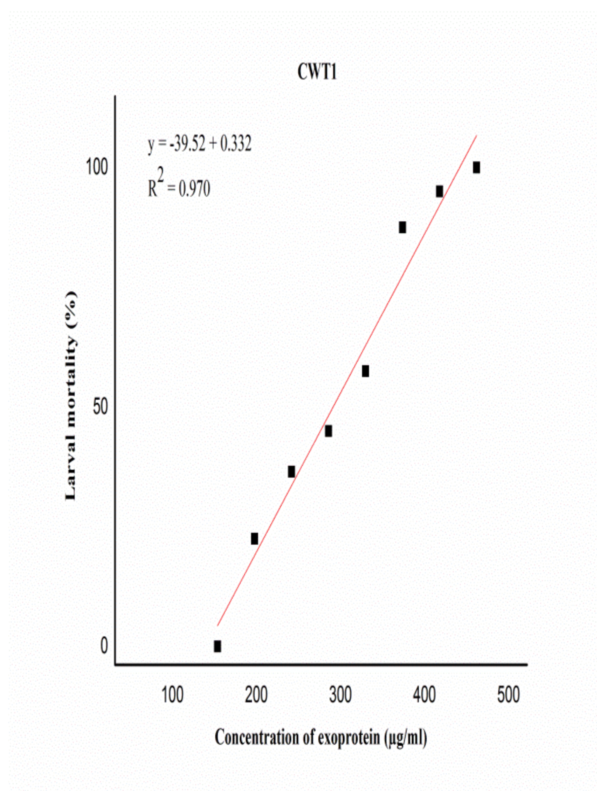
Statistical analysis of the experimental data was analyzed using regression equation (Y= mortality; X= concentration) and corresponding regression coefficient values by using SPSS software. The results of regression analysis reported that the mosquito larval mortality rate (Y) of *Aedes aegypti* was significantly positively correlated with the concentration of bacterial exoproteins of non-agricultural site soil samples with respect to exposure period ($p<0.05$) and the regression coefficient values were calculated for PON2 (R^2 =0.985) in (Fig 5.8).

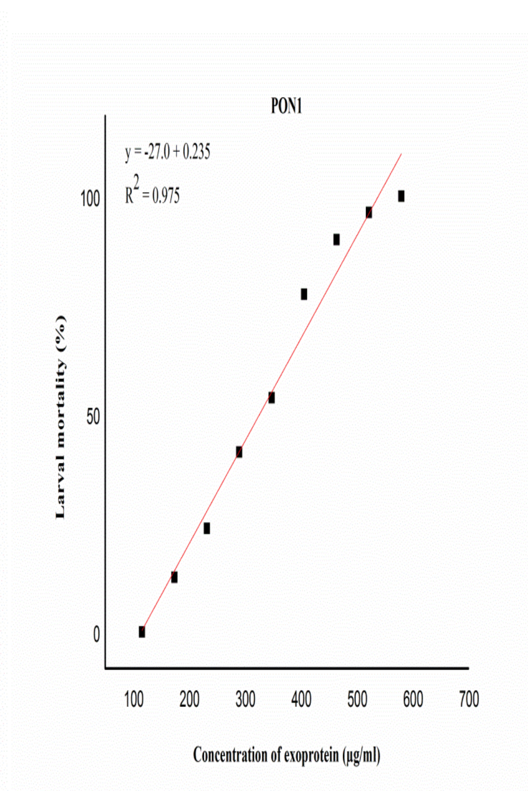
Fig 5.8 Evaluation of biocontrol efficacy of bacterial strains from non-agricultural field samples against third instar larvae of *Aedes aegypti.*

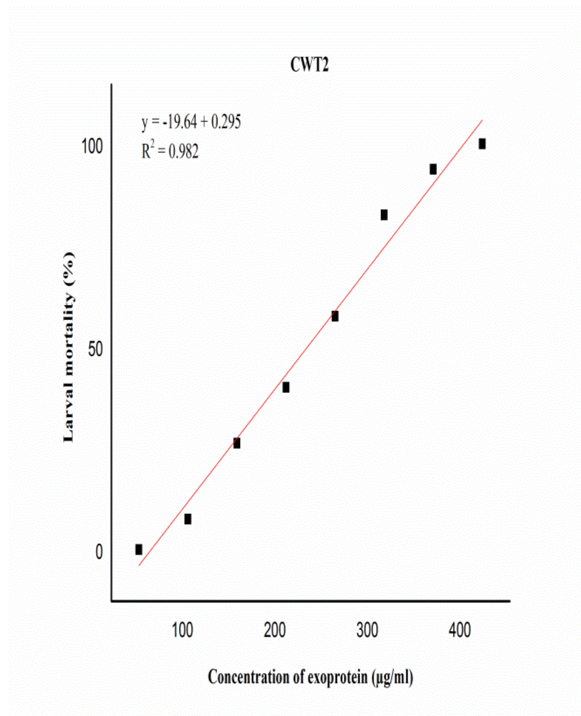


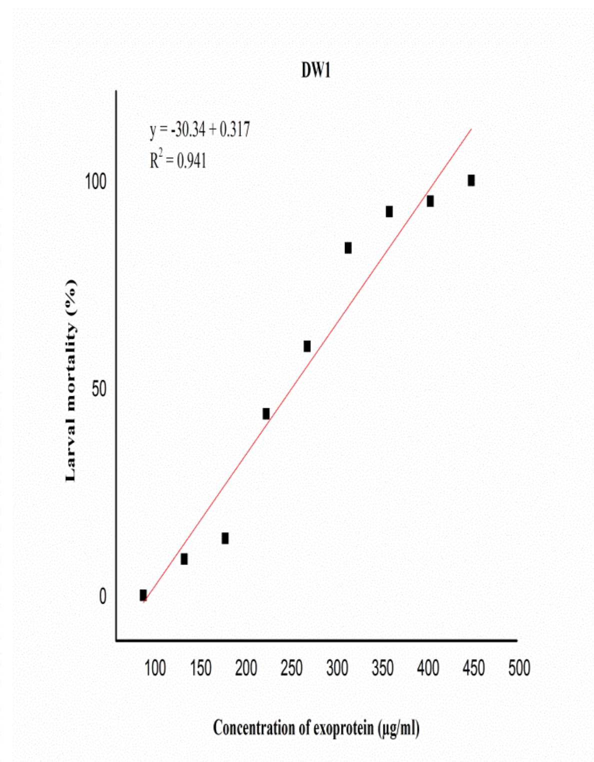

SWY
y = 16.70 + 0.166
R2 = 0.974
Larval mortality (%)
Concentration of exoprotein (µg/ml)

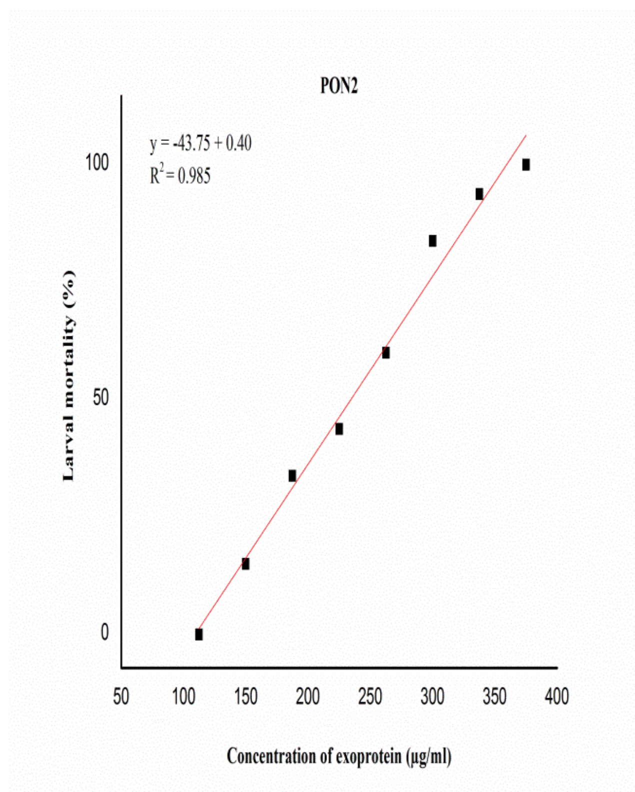

VAG
y = -26.60 + 0.214
R2 = 0.993
Larval mortality (%)
Concentration of exoprotein (µg/ml)


CWT1
y = -39.52 + 0.332
R2 = 0.970
Larval mortality (%)
Concentration of exoprotein (µg/ml)


PON1
y = -27.0 + 0.235
R2 = 0.975
Larval mortality (%)
Concentration of exoprotein (µg/ml)

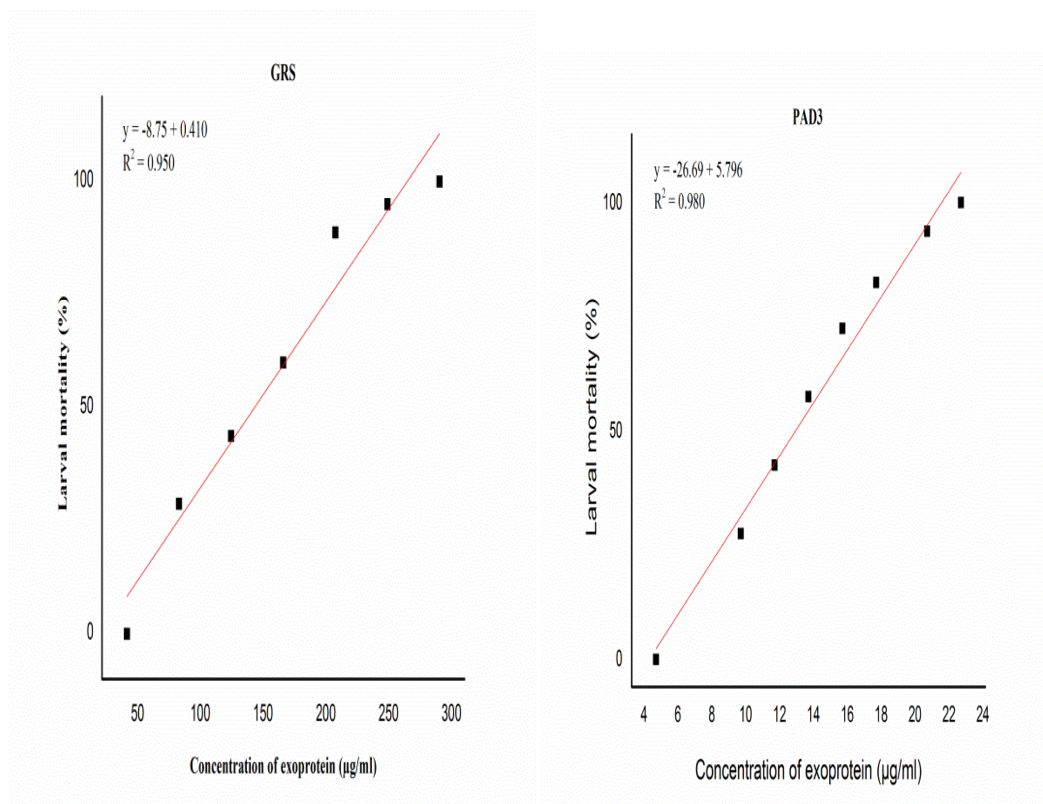

CWT2
y = -19.64 + 0.295
R2 = 0.982
100
50
0
Larval mortality (%)
100
200
300
400
Concentration of exoprotein (µg/ml)

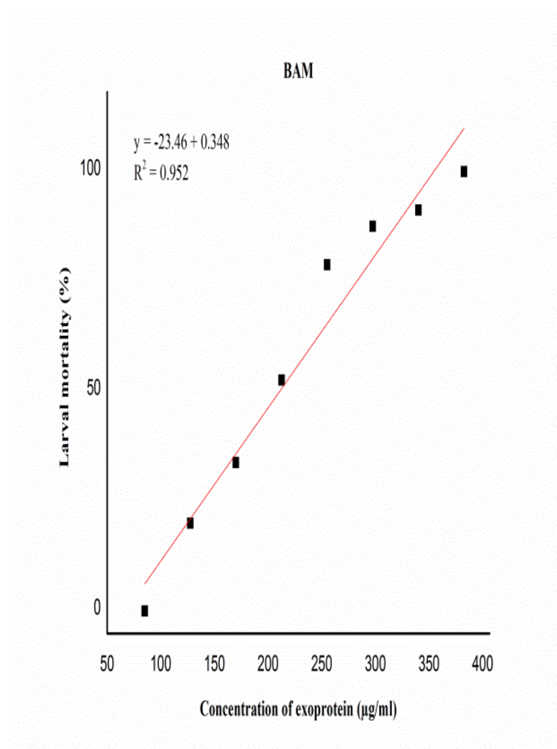

DW1
y = -30.34 + 0.317
R2 = 0.941
100
50
0
Larval mortality (%)
100
150
200
250
300
350
400
450
500
Concentration of exoprotein (µg/ml)

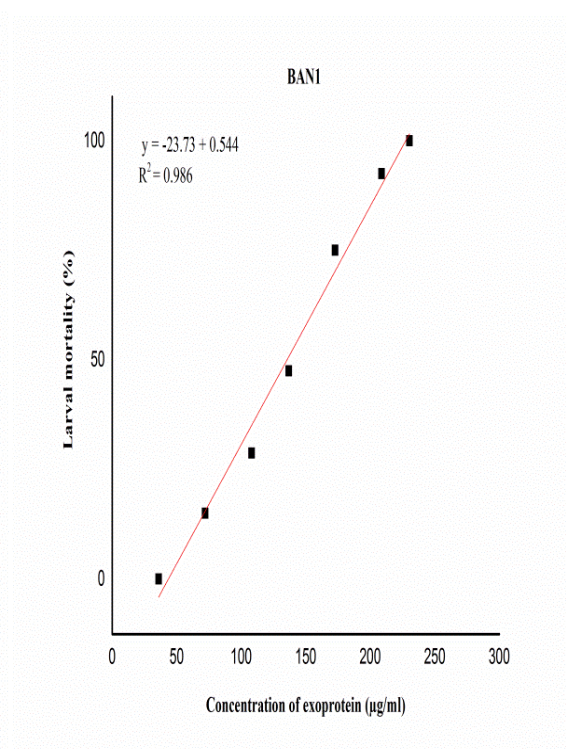

PON2
y = -43.75 + 0.40
R2 = 0.985
100
50
0
Larval mortality (%)
50
100
150
200
250
300
350
400
Concentration of exoprotein (µg/ml)

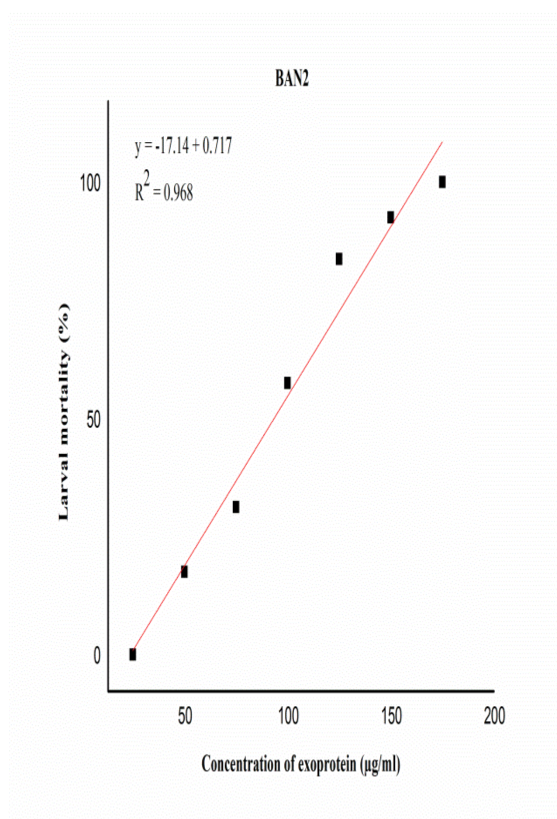
The results of the regression analysis revealed that the mosquito larval mortality rate (Y) was significantly positively correlated with the concentration of bacterial exoproteins with respect to exposure period ($p<0.05$) and the regression coefficient values were calculated as PAD3 (R^2 =0.980), SUG2 (R^2 =0.995), and PAD1 (R^2 =0.990) respectively (Fig 5.9). In this present study, out of twenty bacterial strains screened against the third instar larvae of *Culex quinquefasciatus*, the above three bacterial strains were highly effective in causing mortality of larvae at a minimum concentration of exoproteins.

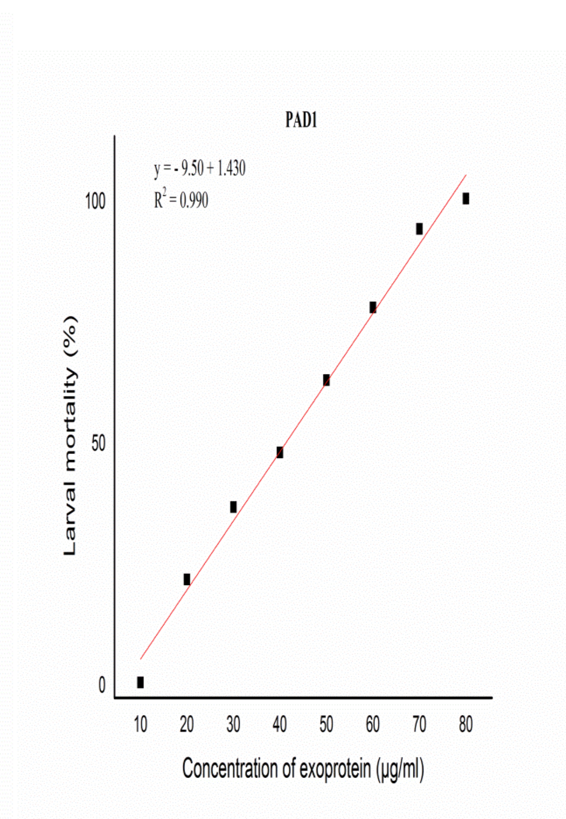
Fig 5.9 Evaluation of biocontrol efficacy of bacterial strains from agricultural field against third instar larvae of mosquito, *Culex quinquefasciatus*

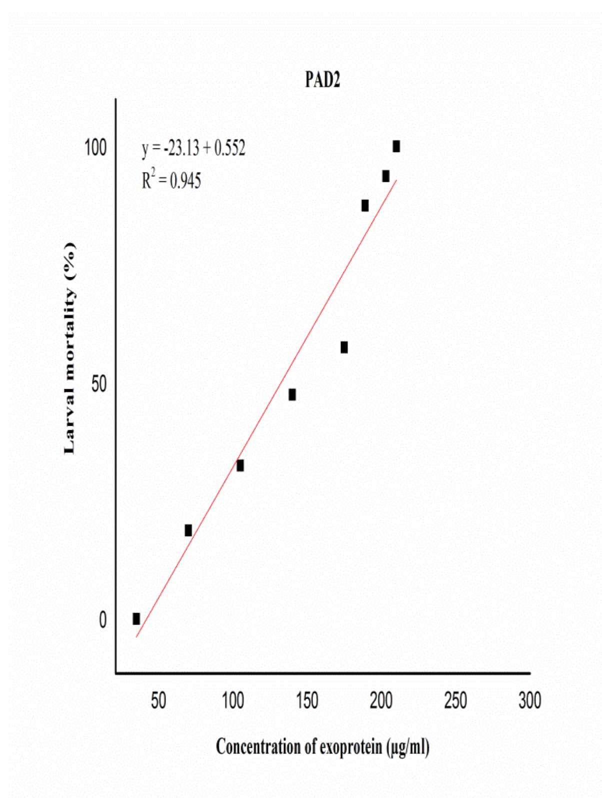


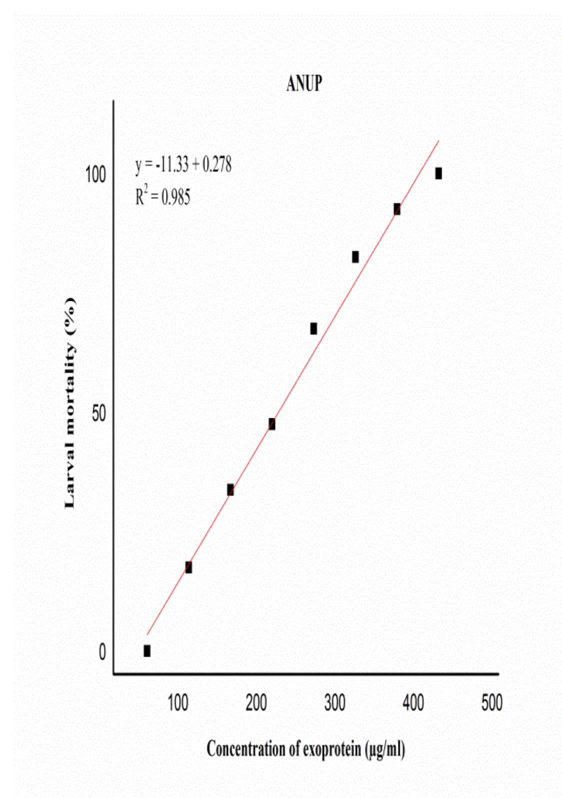

BAM
y = -23.46 + 0.348
R2 = 0.952
Larval mortality (%)
100
50
0
50 100 150 200 250 300 350 400
Concentration of exoprotein (µg/ml)

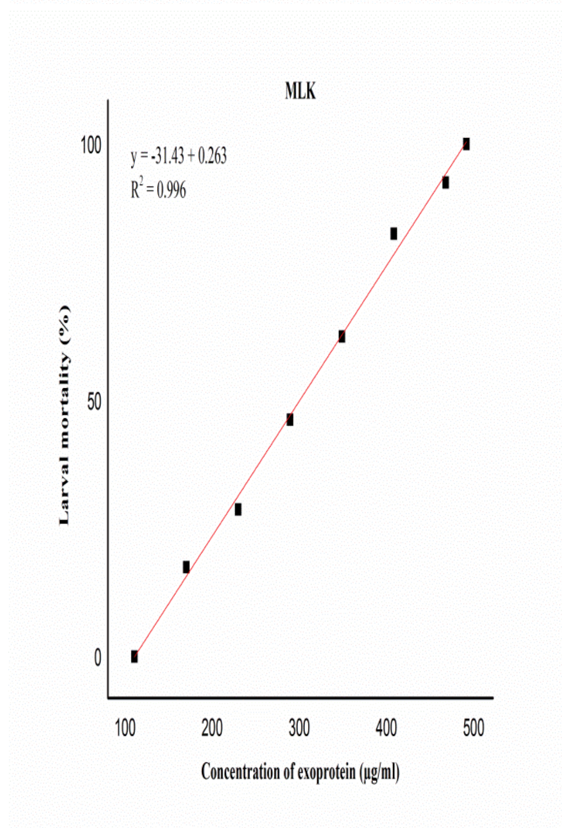

BAN1
y = -23.73 + 0.544
R2 = 0.986
Larval mortality (%)
100
50
0
0 50 100 150 200 250 300
Concentration of exoprotein (µg/ml)

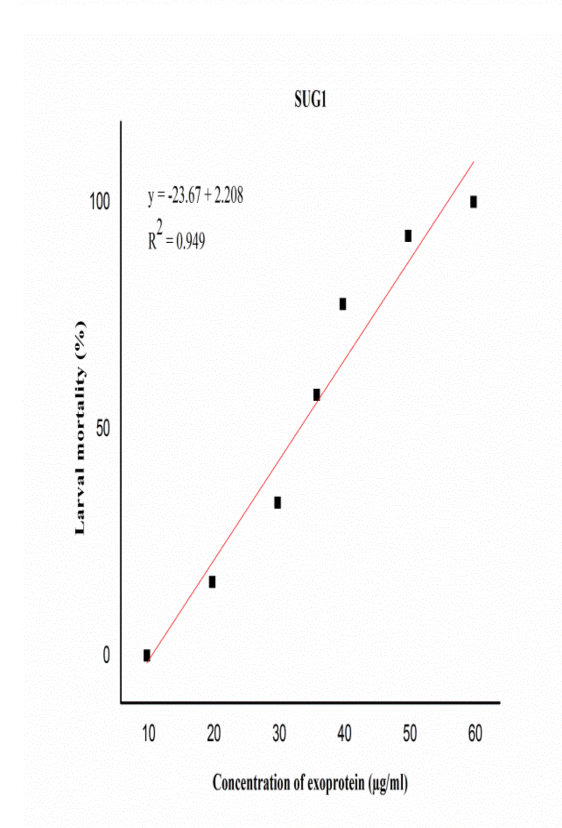

BAN2
y = -17.14 + 0.717
R2 = 0.968
Larval mortality (%)
100
50
0
50 100 150 200
Concentration of exoprotein (µg/ml)

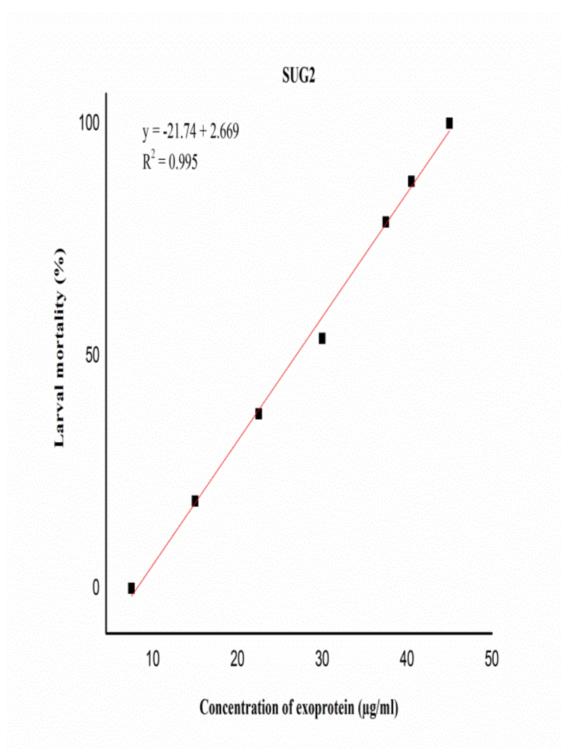

PAD1
y = - 9.50 + 1.430
R2 = 0.990
Larval mortality (%)
100
50
0
10 20 30 40 50 60 70 80
Concentration of exoprotein (µg/ml)


PAD2
y = -23.13 + 0.552
R2 = 0.945
100
50
0
Larval mortality (%)
50
100
150
200
250
300
Concentration of exoprotein (µg/ml)


ANUP
y = -11.33 + 0.278
R2 = 0.985
100
50
0
Larval mortality (%)
100
200
300
400
500
Concentration of exoprotein (µg/ml)

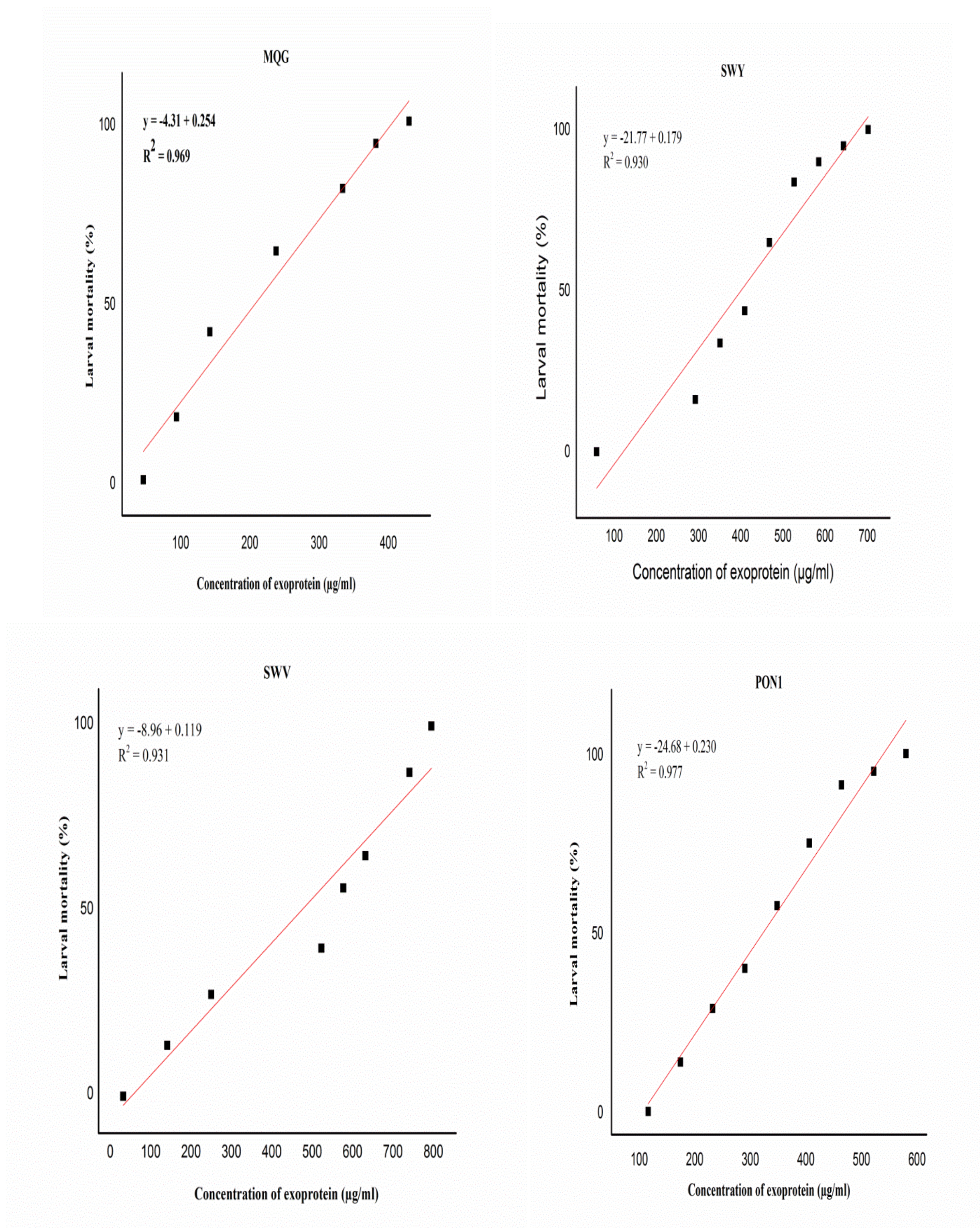

MLK
y = -31.43 + 0.263
R2 = 0.996
100
50
0
Larval mortality (%)
100
200
300
400
500
Concentration of exoprotein (µg/ml)

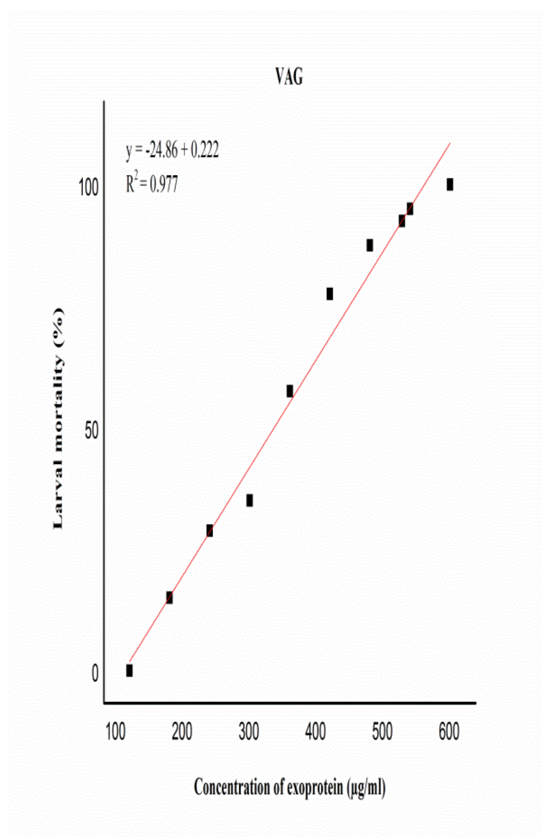

SUG1
y = -23.67 + 2.208
R2 = 0.949
100
50
0
Larval mortality (%)
10
20
30
40
50
60
Concentration of exoprotein (µg/ml)

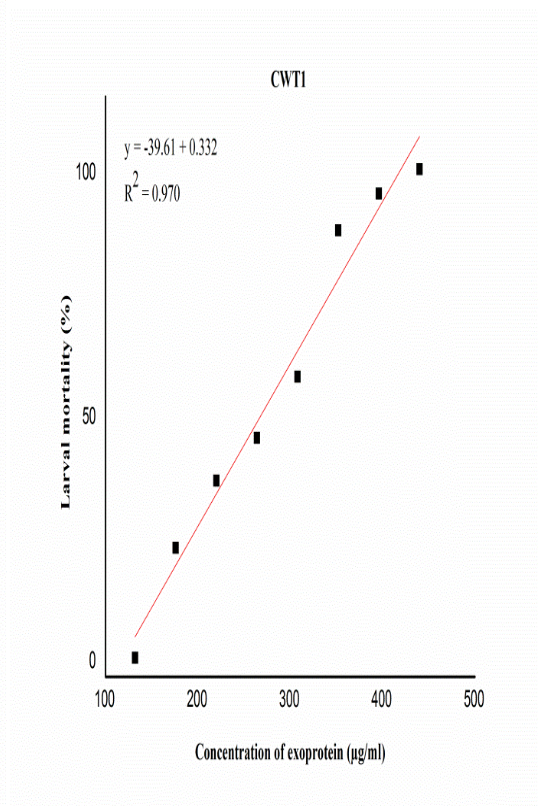


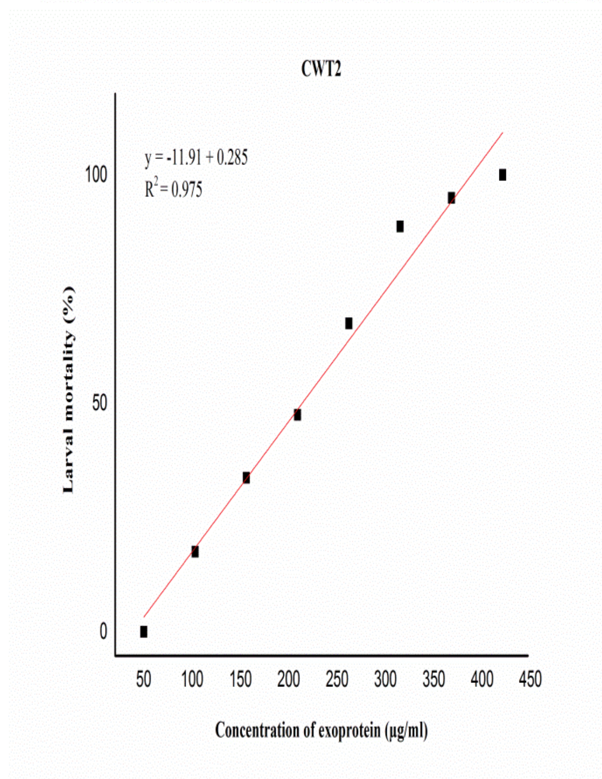
Statistical analysis of the experimental data was analyzed using regression equation (Y= mortality; X= concentration) and corresponding regression coefficient values were calculated. The results of regression analysis reported that the mosquito larval mortality rate (Y) of *Culex quinquefasciatus* was significantly positively correlated with the concentration of bacterial exoproteins of non-agricultural site soil samples with respect to exposure period ($p<0.05$) and the regression coefficient values were calculated for PON2 (R^2 =0.985) in (Fig 5.10).

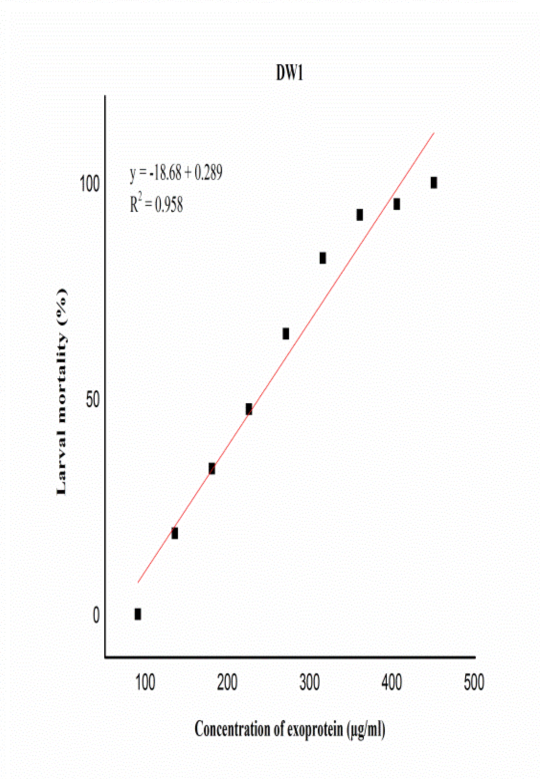
Fig 5.10 Evaluation of Biocontrol efficacy of bacterial strains from non-agricultural field against third instar larvae of mosquito, *Culex quinquefasciatus:*

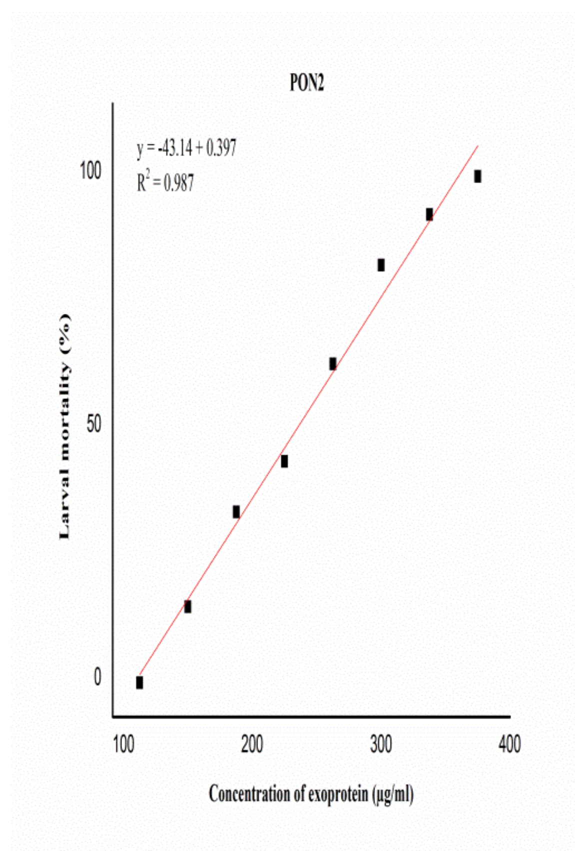



VAG
y = -24.86 + 0.222
R2 = 0.977
100
50
0
Larval mortality (%)
100 200 300 400 500 600
Concentration of exoprotein (µg/ml)


CWT1
y = -39.61 + 0.332
R2 = 0.970
100
50
0
Larval mortality (%)
100 200 300 400 500
Concentration of exoprotein (µg/ml)


CWT2
y = -11.91 + 0.285
R2 = 0.975
100
50
0
Larval mortality (%)
50 100 150 200 250 300 350 400 450
Concentration of exoprotein (µg/ml)


DW1
y = -18.68 + 0.289
R2 = 0.958
100
50
0
Larval mortality (%)
100 200 300 400 500
Concentration of exoprotein (µg/ml)



The results of the regression analysis revealed that the mosquito larval mortality rate (Y) was significantly positively correlated with the concentration of bacterial exoproteins with respect to exposure period ($p<0.05$) and the regression coefficient values were calculated as shown in (Fig 5.10). In this present study, out of nine bacterial strains from non-agricultural fields screened against third instar larvae of *Culex quinquefasciatus*, one strain from the mosquito breeding site (MQG) was highly effective in causing mortality of larvae at a minimum concentration of exoproteins.

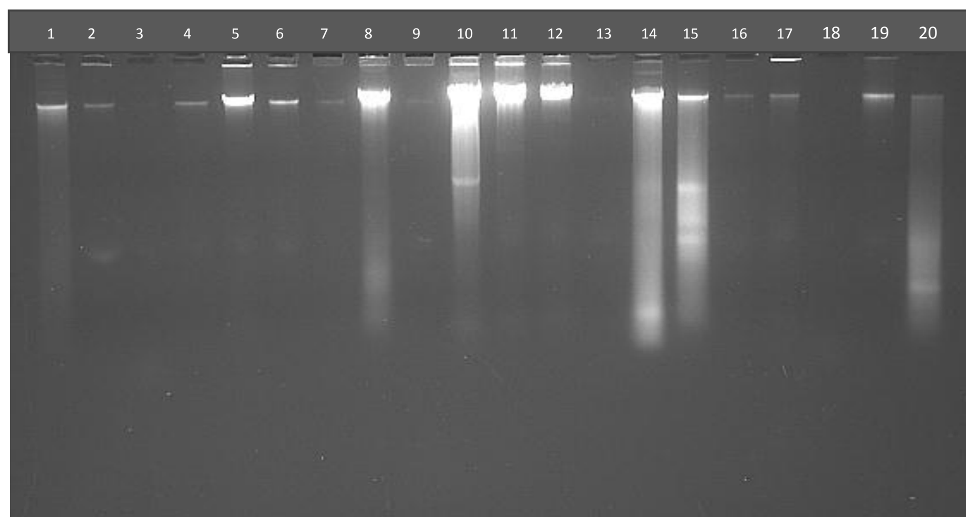
5.5 Molecular characterization of bacterial strains

The ribosomal RNA (r-RNA) gene is particularly important for phylogenetic analysis because it includes highly conserved regions as well as highly variable regions which is used to reconstruct phylogenetic relationships among organisms with varying degrees of relatedness. The morphological and biochemical approach is widely used in bacterial identification. Due to the development of molecular biology techniques, homology analysis based on 16S r-RNA gene sequences has become the "golden index" in the field of bacterial identification.

5.5.1 Isolation of DNA from isolated bacterial strains

The isolated bacterial strains were subjected to molecular characterization for identification. The DNA was isolated from 24 hrs culture of all bacterial strains and amplified the DNA by Polymerase Chain Reaction (PCR). The obtained amplified DNA was subjected to agarose gel electrophoresis and the visible DNA bands were observed on the gel under UV transilluminator. The DNA bands of the isolated bacterial strains were shown in the Fig (5.11).

Fig 5.11 PCR gel picture of the bacterial strains isolated from both agricultural fields and non-agricultural sites



Lane: 1-CWT1, 2-MQG, 3-SWY, 4-PAD1, 5-SWV, 6-BAM, 7-PON1, 8-VAG, 9-BAN1, 10-BAN2, 11-DW1, 12-CWT2, 13-ANUP, 14-GRS, 15-MLK, 16-SUG1, 17-PAD3, 18-PAD2, 19-SUG2, 20-PON2

Agarose gel electrophoresis is used for separating DNA fragments of varying sizes ranging from 100bp to 25kb. In this gel picture shown in fig.5.11, lanes1, 2, 4, 6, 17 and 19 showed discrete bands whereas lanes 7, 9, 13, 16, 20 showed thin bands of less concentration of DNA. Lanes 5, 8, 10, 11, 12, 14, 15 showed thick bands with smear whereas lanes3 and 18 showed no bands.

5.5.2 16S r-RNA gene sequencing and analysis

The 16S r-RNA gene was amplified for nine DNA samples and sequenced for the forward and reverse strands. Comparison of query sequences with the NCBI public data base using Blast similarity search tool. The phylogeny analysis of query

sequence with the closely related sequences based on Blast analysis was performed followed by multiple sequence alignment.

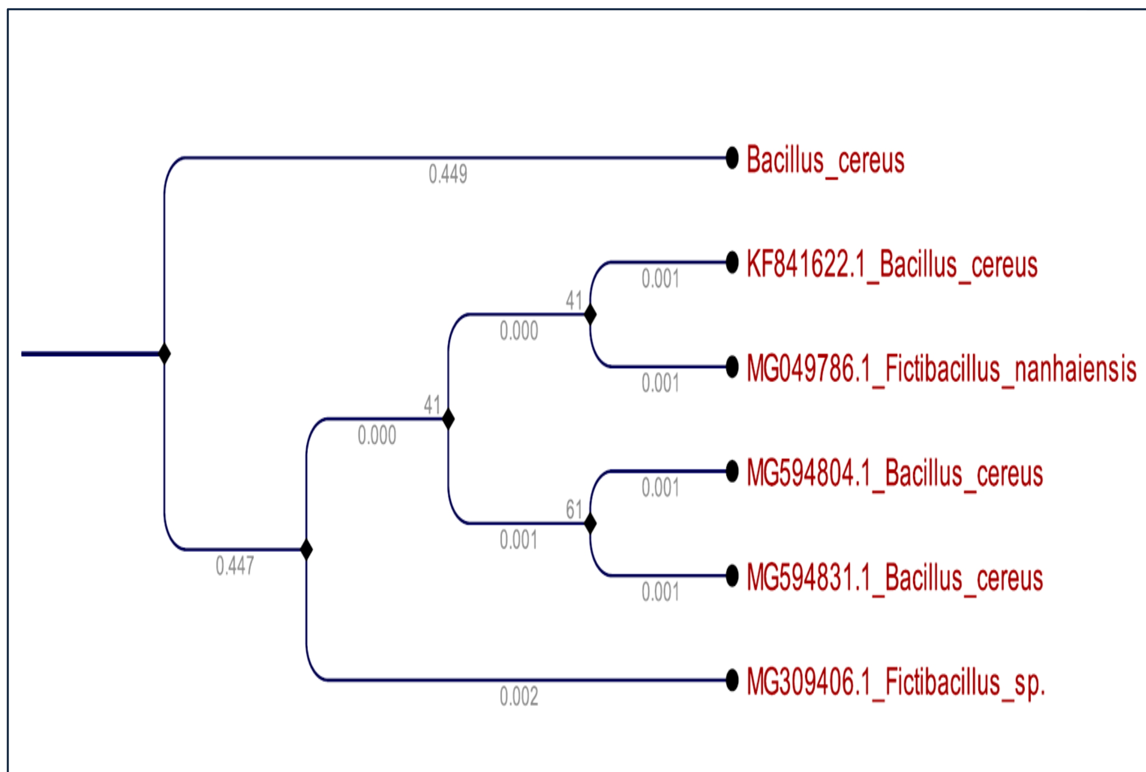
Molecular analysis results indicated that strain CWT1 showed 99% identity with *Enterobacter cloacae*, strain DW1 showed 99% identity with *Bacillus cereus*, strain MQG showed 99% homologous with *Exiguobacterium profundum*, strain PAD1 showed 100% identity with *Pseudomonas aeruginosa*, strain PON1 showed 99% identity with *Acinetobacter baumannii*, strain SUG1 showed 99% identity with *Bacillus cereus*, strain SUG2 showed 95% identity with *Bacillus firmus*, strain PAD3 showed 99% identity with *Bacillus cereus* and strain BAM showed 99% identity with *Cupriviridus taiwensis.* A phylogenetic relationship was investigated using the Neighbour-joining method through the alignment and cladistics analysis of the nucleotide sequences among the bacterial strains.

5.5.3 Phylogenetic tree construction

The morphological, biochemical and gene sequence data suggests that all organisms on Earth are genetically related. This genetic relationship can be studied through phylogenetic tree constructs. In a phylogenetic tree, each node with descendants represents the most recent common ancestor of the descendants and the edge lengths correspond to time estimates. A small region of DNA or protein sequences can be used as input for the computation of phylogenetic trees. It is widely applied in biological classification particularly in bacterial taxonomy.

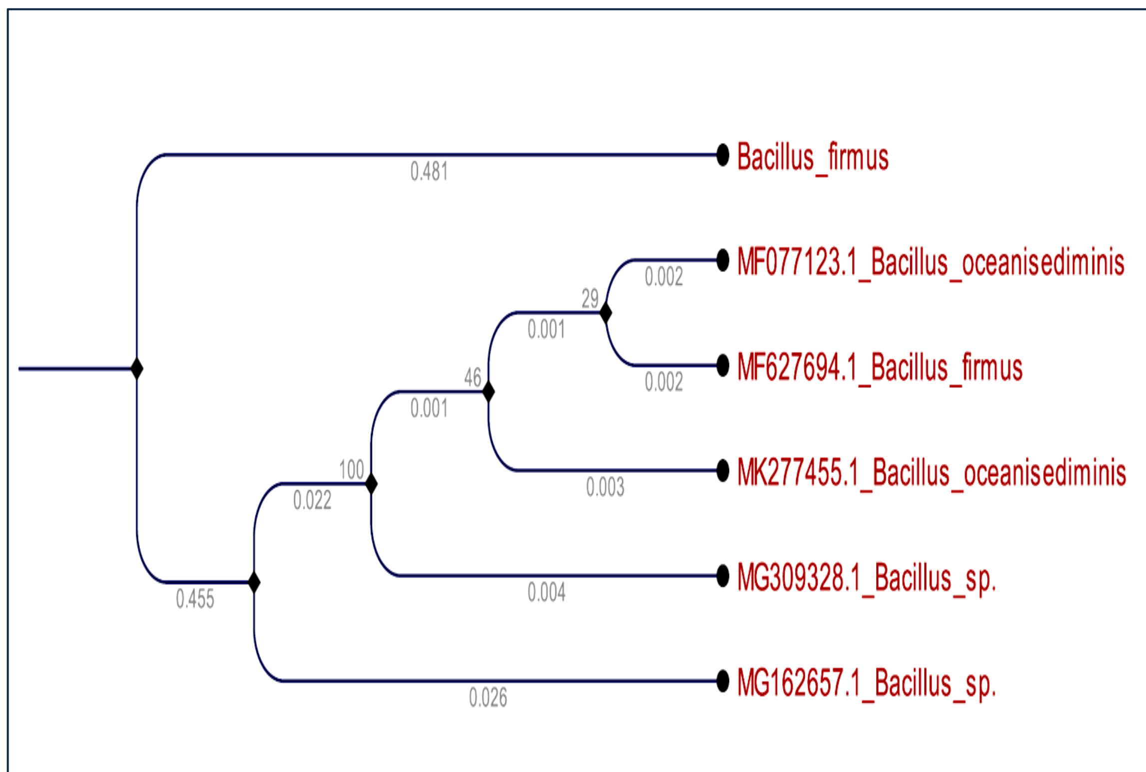
The phylogenetic tree was constructed for each bacterial strain isolated from agricultural and non-agricultural sites, which are effective against mosquito larvae of *Aedes aegypti* and *Culex quinquefasciatus.* The genetic relatedness of these isolated bacterial strains with identical sequences from BLAST results were identified and report

Fig 5.12 Phylogenetic tree showing relationship between bacterial strain PAD3 and closely related organisms.



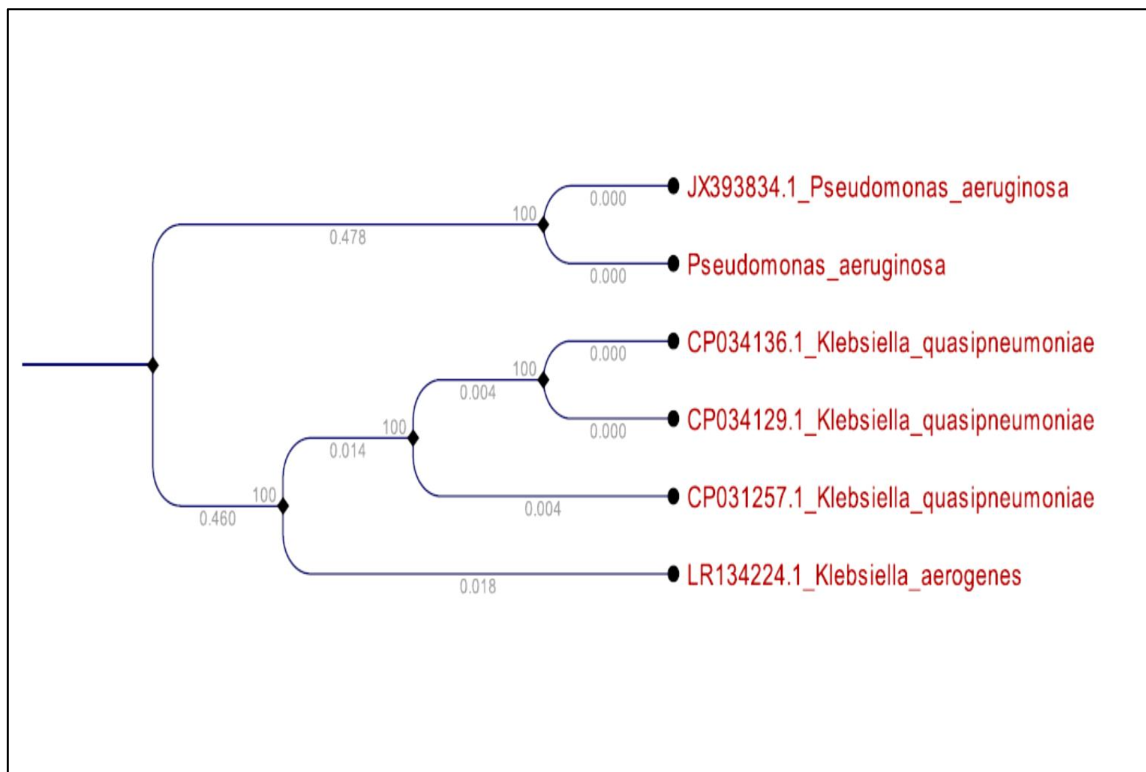
Dendrogram or phylogenetic tree is a branching diagram showing the evolutionary relationship between species. PAD3 strain isolated from paddy rhizosphere soil showed 99% identity with *Bacillus cereus* shown in fig. 5.12. *Bacillus cereus* is a gram-positive, rod shaped, motile, beta-hemolytic bacterium commonly found in soil. It showed close phenotypic and genetic relationships to several other *Bacillus* species.

Fig 5.13 Phylogenetic tree showing relationship between bacterial strain SUG2 and closely related organisms.



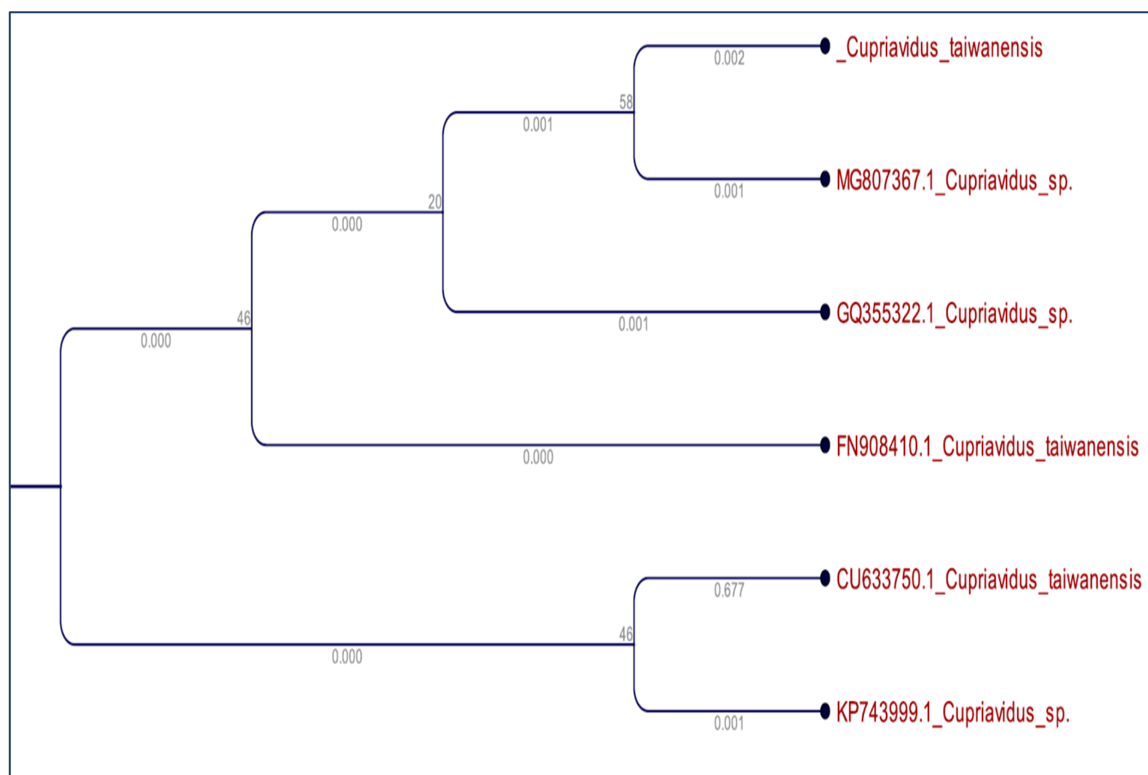
The bacterial strain (SUG2) isolated from sugarcane rhizosphere soil showed 99% identity with *Bacillus firmus* shown in fig.5.13. *Bacillus firmus* is an aerobic, gram positive, spore forming bacterium isolated from diverse environments and widely used in biocontrol of plant pathogens.

Fig 5.14 Phylogenetic tree showing relationship between bacterial strain PAD1 and closely related organisms.



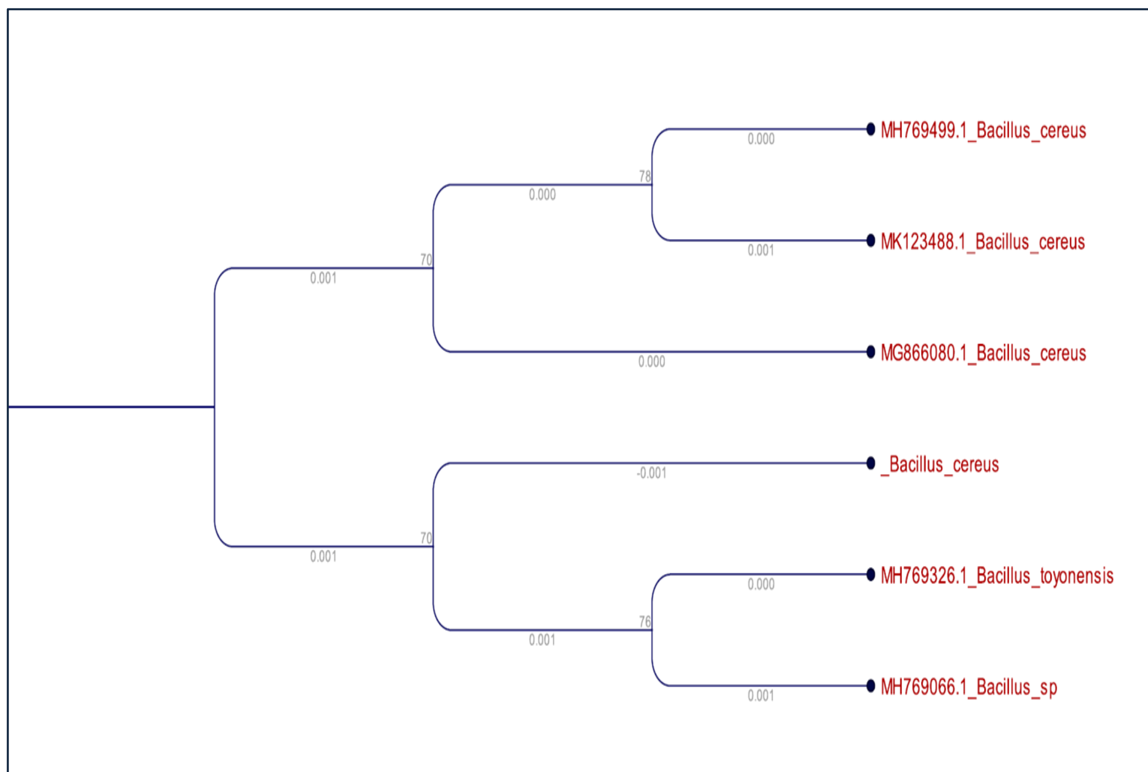
The bacterial strain (PAD1) isolated from paddy rhizosphere soil showed 99% identity with *Pseudomonas aeruginosa* shown in fig.5.14. *Pseudomonas aeruginosa* is a gram-negative, rod-shaped, asporogenous and monoflagellated bacterium, widely distributed in soil and water.

Fig 5.15 Phylogenetic tree showing relationship between bacterial strain BAM and closely related organisms.



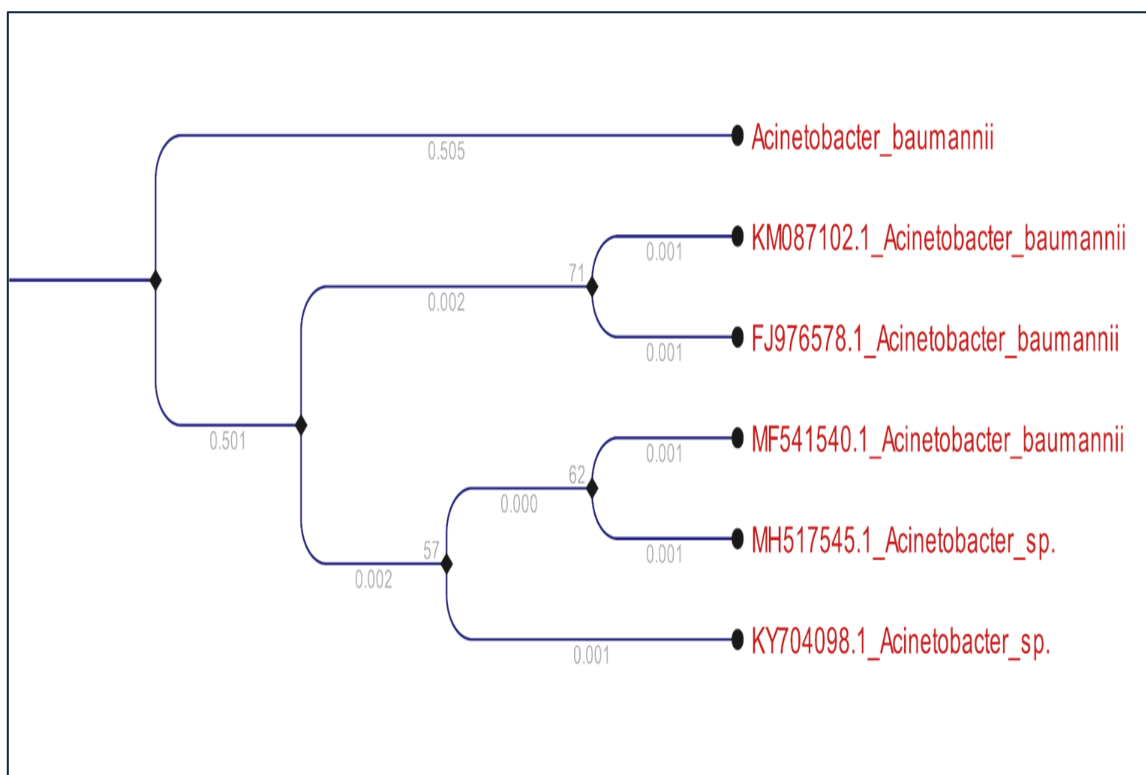
The bacterial strain (BAM) isolated from bamboo rhizosphere soil showed 99% identity with *Cupriavidus taiwanensis* shown in fig.5.15. *Cupriavidus taiwanensis* (originally called Ralstonia taiwanensis or Wautersia taiwanensis), a betaproteobacteria of the rhizobia group, was isolated from a nodule from the legume species Mimosa pudica in Taiwan. They have been isolated from soil, water, plants, rhizosphere and insects (Burkholderia).

Fig 5.16 Phylogenetic tree showing relationship between bacterial strain SUG1 and closely related organisms.



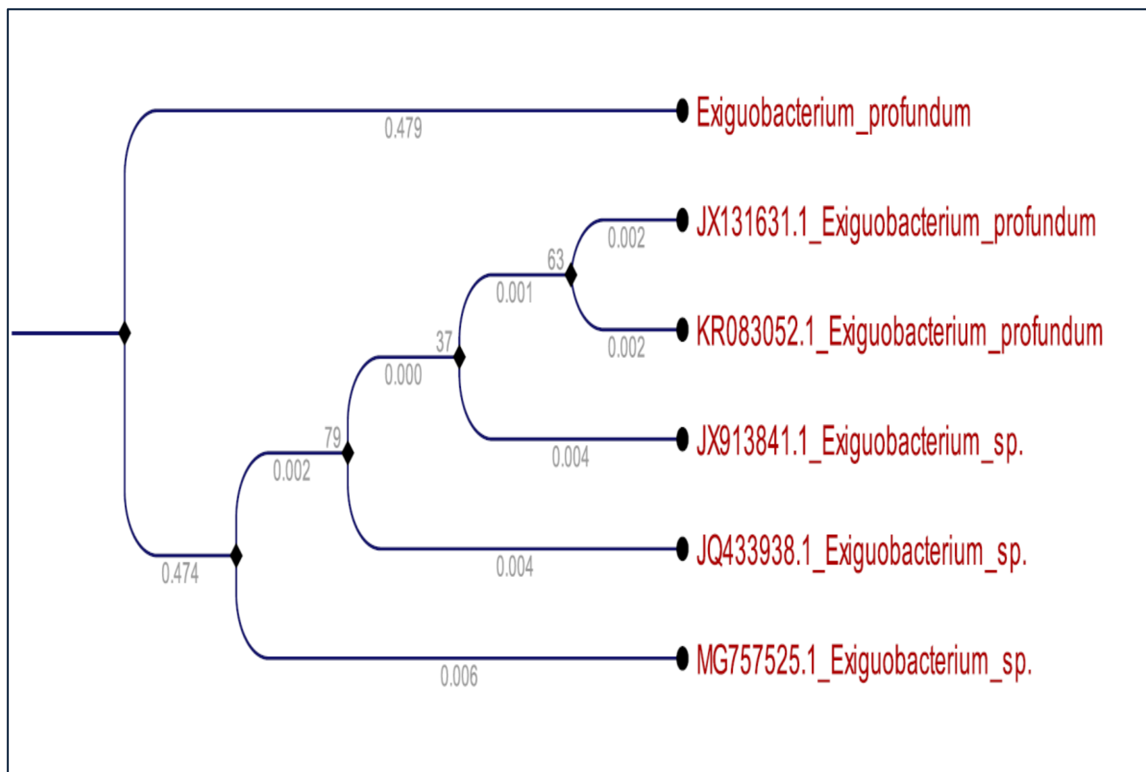
The bacterial strain (SUG1) isolated from sugarcane rhizosphere soil showed 99% identity with *Bacillus cereus* shown in fig.5.16. *Bacillus cereus* is a gram-positive, rod shaped, motile, beta-hemolytic bacterium commonly found in soil. It showed close phenotypic and genetic relationships to several other *Bacillus* species.

Fig 5.17 Phylogenetic tree showing relationship between bacterial strain MQG and closely related organisms.



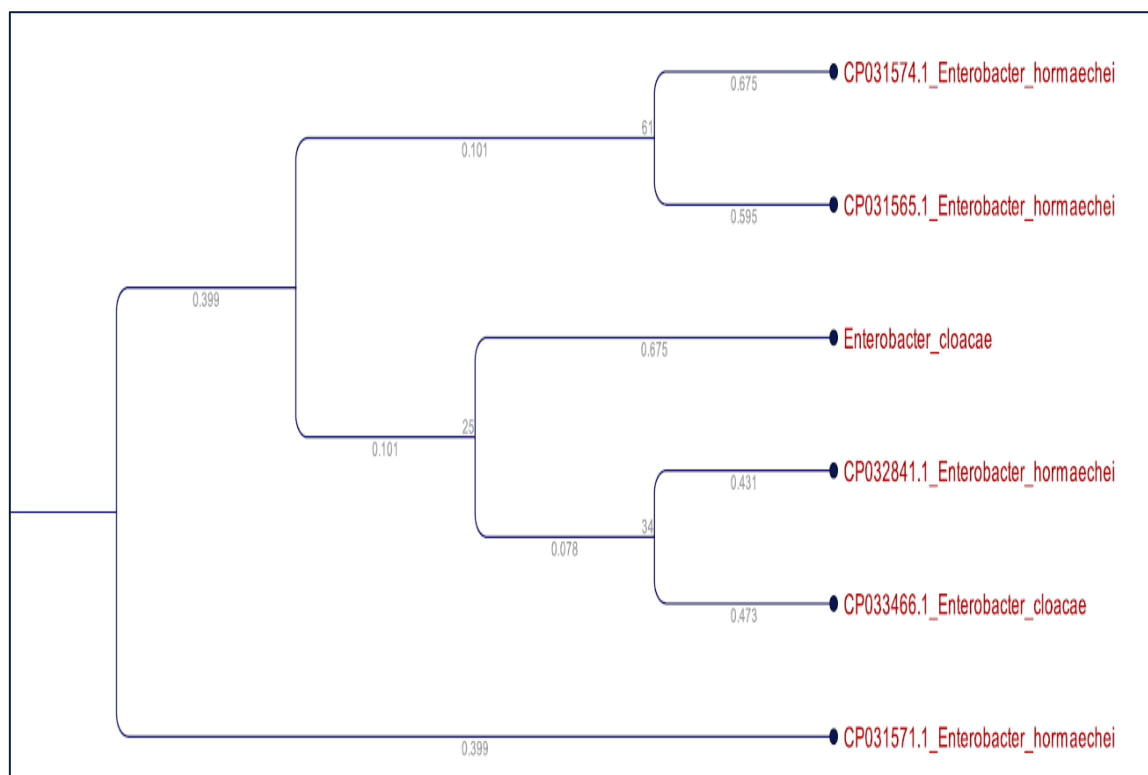
The bacterial strain (MQG) isolated from mosquito breeding site sediment showed 99% identity with *Acinetobacter baumannii* shown in fig.5.17. *Acinetobacter baumannii*, an obligate aerobic bacterium commonly found in soil, water and sewage. It showed close phenotypic and genetic relationships to several other species belong to Moraxellaceae family.

Fig 5.18 Phylogenetic tree showing relationship between bacterial strain PON1 and closely related organisms.



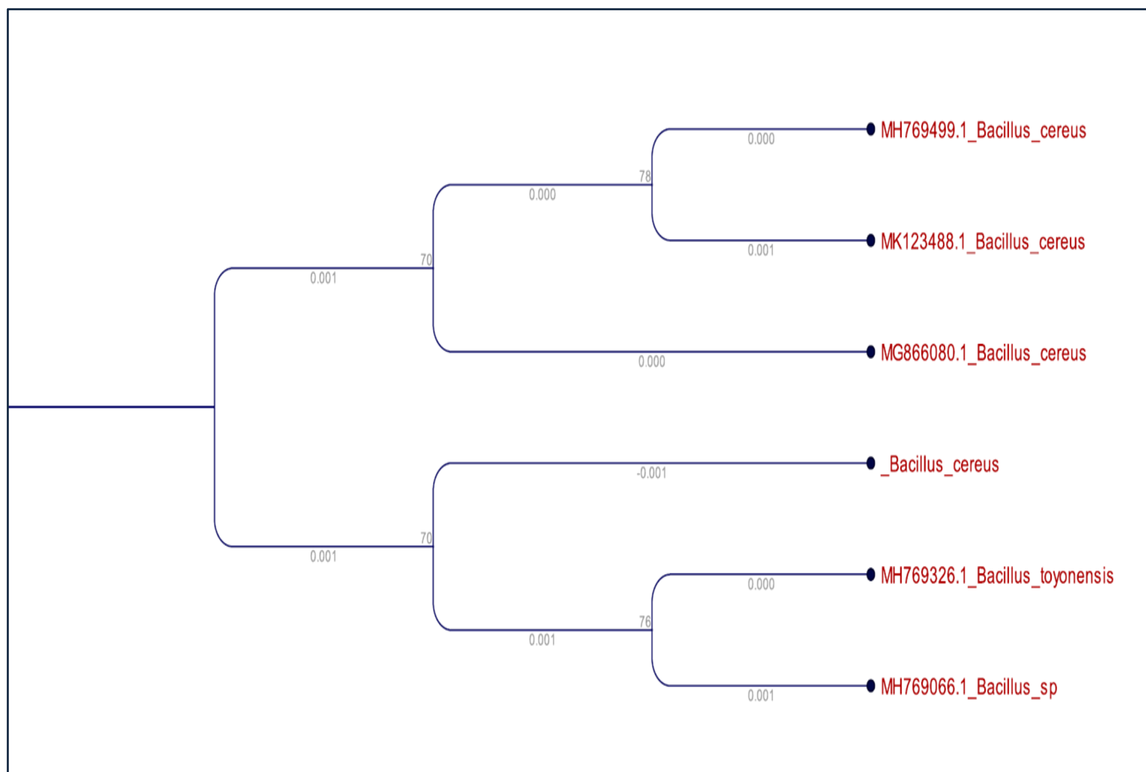
The bacterial strain (PON1) isolated from pond sediment showed 99% identity with *Exiguobacterium profundum* shown in fig.5.18. *Exiguobacterium* is a rod-shaped, mesophilic gram-positive, facultative anaerobic bacterium belongs to member of Firmicutes.

Fig 5.19 Phylogenetic tree showing relationship between bacterial strain CWT1 and closely related organisms.



The bacterial strain (CWT1) isolated from Irrigation canal sediment showed 99% identity with *Enterobacter homaechei* shown in fig.5.19. *Enterobacter hormaechei* is a species of gram-negative bacterium, belonging to the *Enterobacter cloacae complex* (ECC) which can exist in the intestinal tract of humans and animals. It belongs to family Enterobactriaceae.

Fig 5.20 Phylogenetic tree showing relationship between bacterial strain DW1and closely related organisms.



The bacterial strain (DW1) isolated from sewage sediment showed 99% identity with *Bacillus cereus* shown in fig.5.20. *Bacillus cereus* is a gram-positive aerobic, motile, non-sporing bacterium which is closely related with the several species belongs to Firmicutes.

5.6 Identification of morphological changes in mosquito larvae treated with exoproteins of bacterial strains

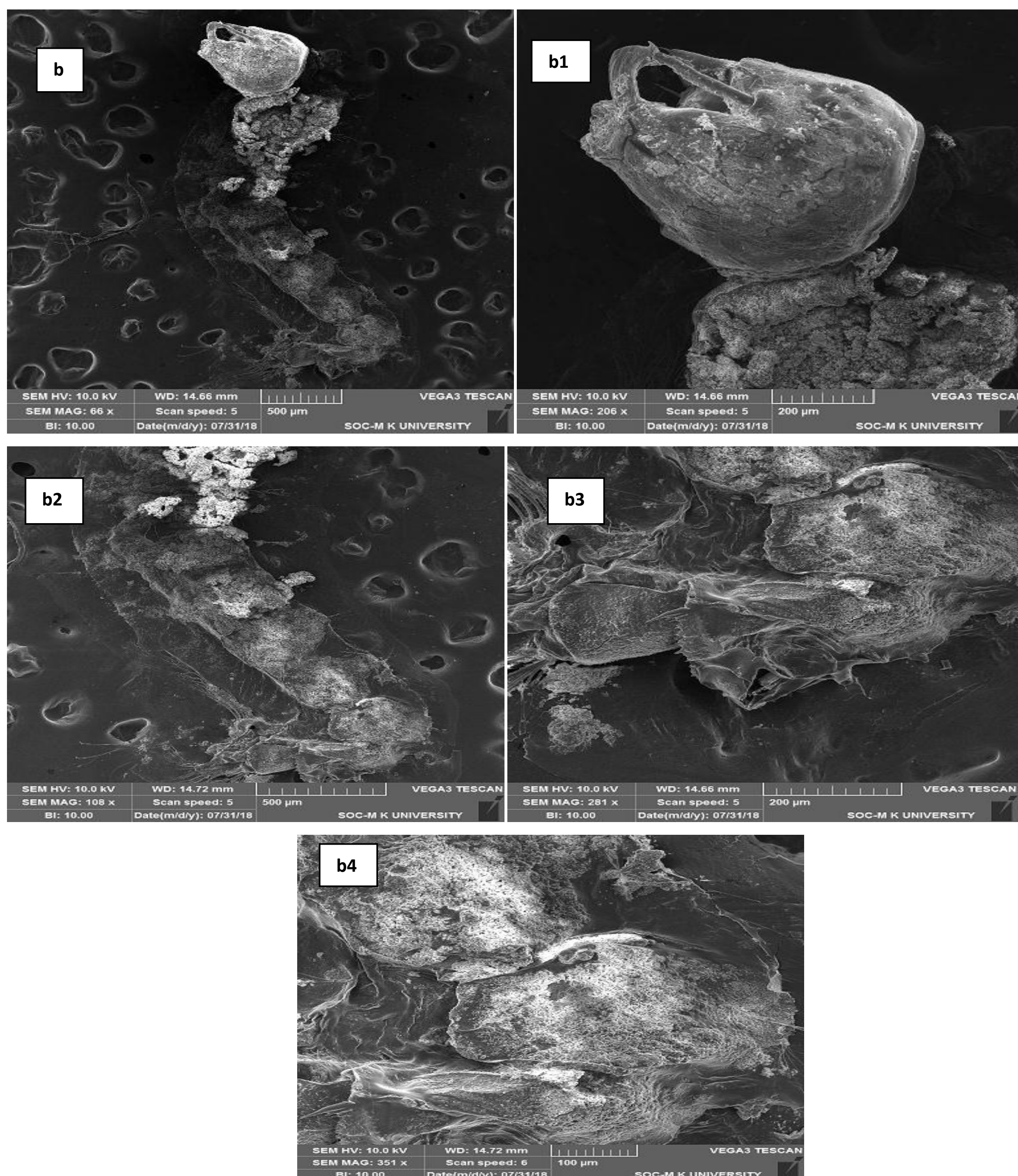
The mosquito vectors, *Aedes aegypti* and *Culex quinquefasciatus* were tested with bacterial strains for mosquitocidal activity. The larvae of both mosquito species were found dead after 24 hrs treatment with exoproteins from both bacterial strains. The observable morphological changes in the dead larvae were observed under a scanning electron microscope

5.6.1 Scanning Electron micrography of *Aedes aegypti* larvae

In this present study, both control and treated larvae of *Aedes aegypti* at 24 hrs post-treatment with exoproteins of highly effective strain *Bacillus cereus* were observed under Scanning Electron microscopy. The results indicated that there was clear and distinct disintegration of body parts of treated larva compared with control larva shown in the Fig.5.22.

Fig 5.21 SEM analysis picture of *Aedes aegypti* larvae (a- control larva untreated, a1- head region of larva, a2 and a3- enlarged view of head region, a4- midgut region, a5- enlarged view of midgut region, a6- enlarged view of hind gut)

Fig 5.22 SEM analysis picture of *Aedes aegypti* larvae (b) treated larvae with exoproteins of *Bacillus cereus* b1 –head region, b2- midgut region showing disintegration, b3- enlarged view of midgut region, b4- enlarged view of hindgut region



But the head region remain intact without any physiological changes in both control and treated larvae (Fig 5.21). Thus, the bacterial toxin act upon ingestion which caused damage in the abdomen region lead to death of the mosquito larvae.

5.6.2 Scanning Electron micrography of *Culex quinquefasciatus* larvae

Both control and experiment larvae of *Culex quinquefasciatus* at 24 hrs post-treatment with exoproteins of highly effective strain *Bacillus cereus* were observed under Scanning Electron microscopy. The SEM pictures indicated that the larva remains intact without any deformations in control shown in Fig. 5.23, but in bacterial exotoxin treated larva showed shrinkage of the body parts. Particularly, the thorax and abdomen regions were completely distorted, but the head region remains intact without any physiological changes (Fig 5.24). Thus, the bacterial toxin caused damage in the abdomen region which leads to death of the mosquito larvae.

Fig 5.23 SEM analysis picture of *Culex quinquefasciatus* larvae (c-control untreated larva, c1-head region of larva, c2- enlarged view of head region, c3- midgut region, c4- enlarged view of hindgut region

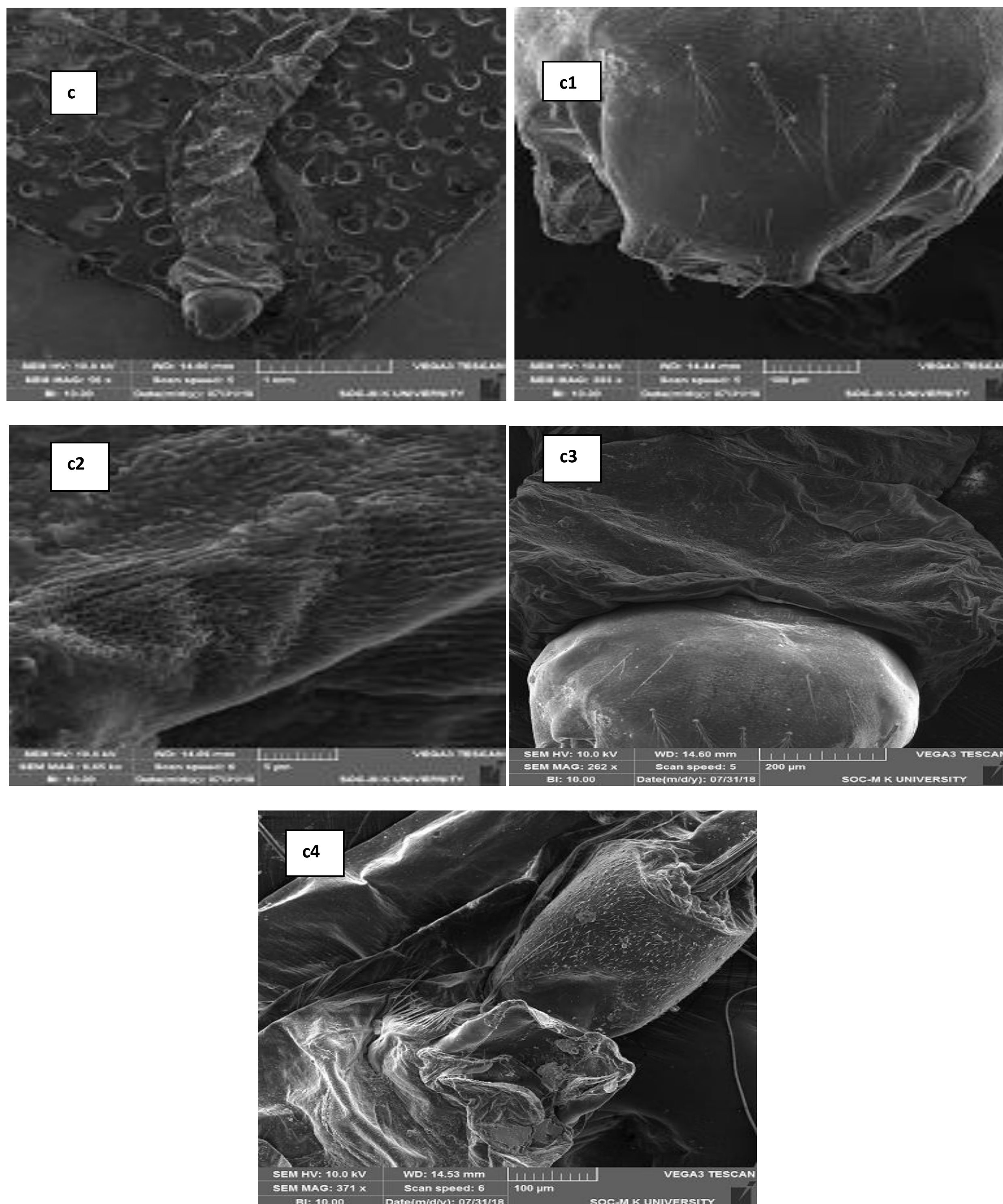
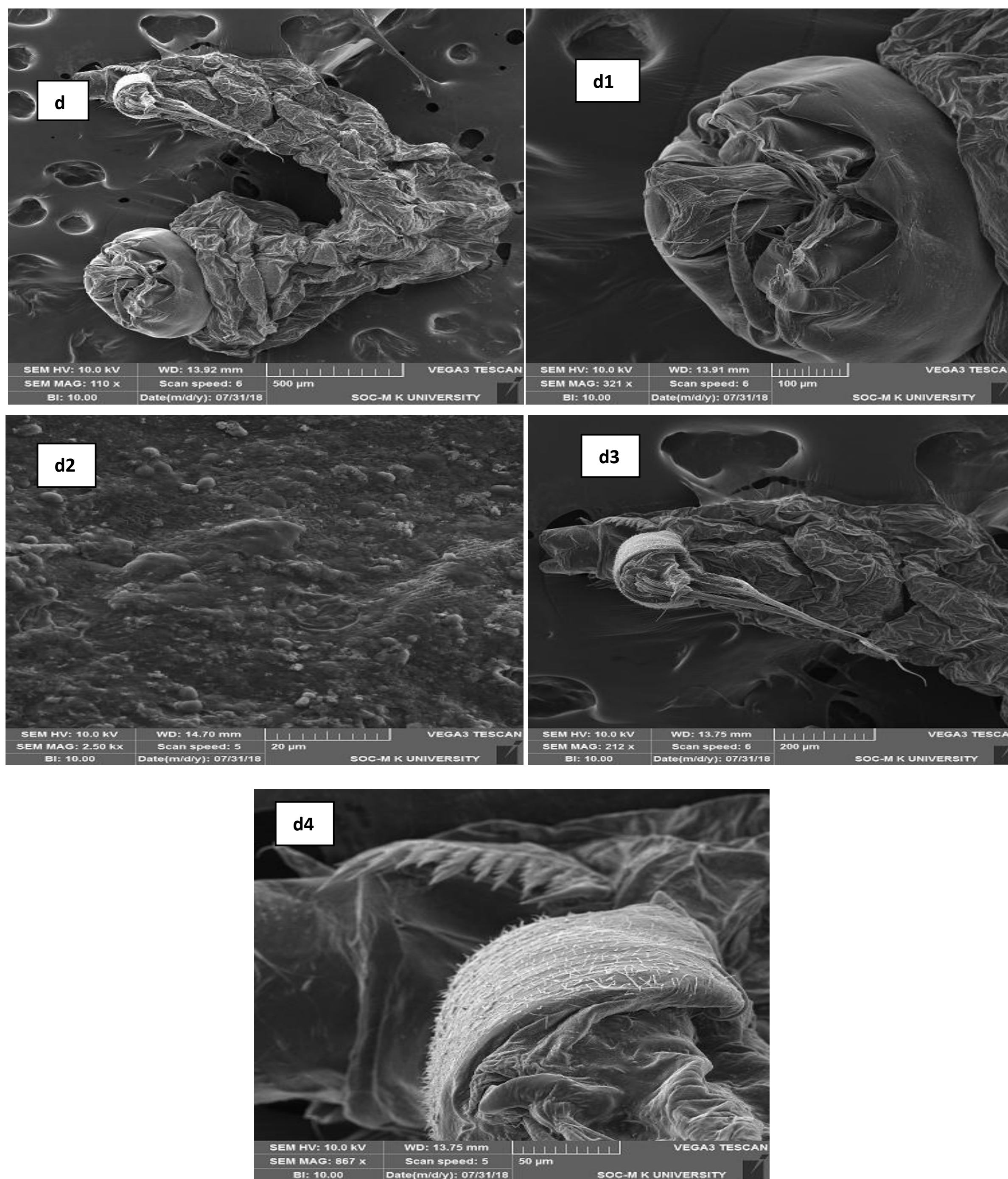


Fig 5.24 SEM analysis of *Culex quinquefasciatus* larva (d- treated larva with exoproteins of *Bacillus cereus,* d1-head region of larva, d2- enlarged view of midgut region showing shrinkage, d3-hindgut region of larva, d4- enlarged view of hindgut.



Discussion

6.0 Discussion

6.1 Physicochemical properties of collected soil samples

Soil as a medium for not only plants and animals but also for various microbes coexists. This viable conditions are influenced by physicochemical properties of soil. Hence, soil chemical indicators hold a link between the physicochemical properties and fertility or productivity of the soil. The various chemical reactions in soil that maintain soil pH, electrical conductivity and organic matter content are indispensable for sustaining soil quality with relation to bacterial density. In the present study, bacterial population in soil samples collected from both agricultural and non-agricultural sites showed different microbial population densities, depend upon various soil types, nutrients availability, variety of growing plant species, *etc*. These characteristic features influence upon type of bacterial community to be present in the soil ecosystem.

6.1.1 Rhizosphere soils

Many plants in its roots having rhizosphere make the dynamic environment. The plant root having exudates contain compounds that selectively support the growth of certain microbial population to colonize in the rhizosphere zone (Nannipeiri *et al*., 2003). In this present study, soil samples bound with various crops having rhizosphere such as from paddy, banana, sugarcane, bamboo were analyzed for bacterial population density. Among these, paddy rhizosphere soil showed higher bacterial density (0.8 $x10^9$ CFU/g) bcause it grows in wet land ecosystem that store a

substantial amount of carbon and moisture, it might have a higher microbial population. Usually, wetland ecosystem consisted of a higher microbial biomass, particularly bacteria, actinomycetes and fungi (Li *et al*., 2017). Among the bacterial population, Bacilli and Clostridia are often highly abundant in paddy agricultural soils. These organisms help decomposition of plant residues using cellulolytic enzymes (Koek *et al*., 2014). Rangarajan *et al*.,(2001) reported that the bacterial strains isolated from the paddy rhizosphere were found to be capable of utilizing more substrates and of being resistant to more antibiotics compared with agricultural crops.

In non-agricultural sites, soil samples were taken from mosquito breeding sites, sewage sediments, pond sediment, and irrigation canal sediment and analyzed for bacterial density in relation to their physicochemical properties. Larval habitats usually are natural or derived from humanmade activities. They are rich in nutrients by which larvae feed on microbial fauna such as bacteria and algae which influence larval productivity. The physicochemical properties of larval habitats largely differ from natural sediment habitats like pond sediment. Ultimately, this results in the change in bacterial density among non-agricultural soil samples (Kumar and Rai, 2017).

6.1.2 pH

Soil pH is often identified as the principal indicator of the chemical characteristic of a particular type of soil. It plays a significant role in all biogeochemical processes and in microbial and enzymatic activity in the soil (Brady and Weil, 2002, Sinsabaugh, 2008). In this present study, pH of all agricultural soil

samples was observed and it ranges from 6.2 to 7.2. Comparatively, bamboo rhizosphere soil has a lower pH (6.2), sandy in texture, organic carbon content (1.20%), which showed a lower bacterial count (0.2 x 10^8 CFU/g) when compared to other soil samples. In bamboo forests, soil pH remains lower than soil from barren land due to the release of protons by root organic anions such as malate, citrate, and oxalate. These are characterized predominantly by sandy soil texture (more than 70% sand and less than 15% clay), high permeability, low water-holding capacity, slow chemical weathering rates, and minimal nutrient content (Mengel, 1994). In contrast, Animal fodder farm soil reported with pH 7.2 showed a bacterial count of (0.4x10^8 CFU/g) which remains higher than bamboo rhizosphere soil. Hence, the neutral pH favors the optimum growth of bacteria.

The pH indicates the solubility of nutrients in the soil which remain essential for the utilization of soil substrates by soil bacteria. The pH ranges from 6.5 to 7.5 in all non-agricultural site samples. Among these, mosquito breeding sites soil prefers 7.5, which is optimum for the growth of bacteria. It showed the highest bacterial density (2.5x10^9 CFU/g) than other sites. This indicated that pH plays a major role in nutrient availability to bacterial population in an environment (Mala and Iringu, 2011). During the utilization of nutrients by bacteria in the soil, the pH decreases due to the accumulation of organic acids reflecting a high organic matter degradation. These acids are used later on as substrate by other microorganisms. During the cooling down and maturation stages the pH changes to a neutral value, which indicates the stabilization of bacterial communities in the soil (Pathak *et al*., 2012).

6.1.3 Organic carbon content

Soil organic matter is an essential parameter in the functioning of the agricultural ecosystem, its productivity and maintain the global carbon cycle (Loveland and Webb, 2003, Pan *et al*., 2009). In the present study, different rhizosphere soil samples from agricultural field such as paddy, sugarcane, banana, bamboo, Garden, cattle fodder farm and vegetable farm were analyzed and it ranges from 5% to 15%. In paddy rhizosphere soil, organic content showed the highest percentage of 15% compared to other soil samples.

The organic soil texture greatly influence with bacterial population where smaller size fractions (slit and clay) harbor higher bacterial community than larger size particles (sand). Organic carbon (OC) present in the soil through the decomposition of plant and animal residues, root exudates, living and dead microorganisms and soil biota. Soil microorganisms use organic matter as a source of energy.

In this present study, both vegetable farm and animal fodder farm enriched with organic carbon content of 4.5% and 4% showed higher bacterial count (0.5 x 10^8 and 0.4 x 10^8) respectively. Similar results were observed due to long- term organic compost application in agricultural farms, which showed increased heterotrophic bacterial growth and improved soil fertility (Benzarti *et al*., 2007 and Gill *et al*., 2016).

There was an input of a relatively small range of readily utilized substrates (proteins, carbohydrates, lipids) in organic polluted sediment soil, which sustained higher bacterial biomass compared to the natural sediment. In this present study, organic carbon content concentration varies from 1.5% to 32%. The sewage sediment soil sample showed a higher bacterial count (2.5 x 10^9 CFU/g) than agricultural soils. Trosvik *et al*., 1996 reported that the presence of organic pollutants in the sewage showed a higher bacterial population with less diversity. This is mainly due to organic substrates exerted a selection pressure favoring a few fast-growing bacteria (r-selection), which become dominant in that particular environment.

Recently, there is an increasing interest in applying organic waste such as sewage sludge and compost as an organic source to agricultural soil. Their application improves organic matter, essential nutrient contents, soil structure, aeration, water holding capacity and microbial activities in soil (Webber *et al*., 1996). However, the decreased beneficial effect of sewage sludge may be due to the toxicity of undesirable materials such as heavy metals present in sludge (Pattanayak *et al*., 2001). The clay fraction has a more diverse bacterial community than silt or sand fractions. This is evident in sludge-impacted soil because it yielded more bacterial diversity due to its high clay fraction. The particle size has a negative impact on microbial biomass density and fine-textured sediment protects microbial biomass. Thus, clay particles are conducive for the formation of biofilms in sediments (Russo *et al*., 2012; Luo *et al*., 2017)

6.1.4 Electrical conductivity

Soil electrical conductivity acts as a measurement of soluble nutrients and it is useful in monitoring the mineralization of organic matter in the soil. The electrical conductivity is an important soil parameter that acts as a direct measurement of salinity and indirectly indicates the total concentration of soluble salts. Electrical conductivity showed an increasing trend with the integrated use of inorganic fertilizer with compost and sewage sludge in the soil. In the present study, the electrical conductivity was increased in both cattle fodder farm and vegetable farm supplemented with organic compost such as (ANUP strain-0.65 and MLK strain-0.80). This may be due to acid or acid- forming compounds that react with soluble salts present in soil, which increased their solubility (Sarwar *et al*., 2003). Comparatively, paddy rhizosphere soils showed higher electrical conductivity value as 0.15 when compared to other samples of rhizosphere soils. This indicated its correlation with organic carbon content and bacterial density.

Electrical conductivity refers to the exchangeable capacity of cations and anions present in a soil sample. In the present study, sewage sediment showed higher (0.28 µS) electrical conductivity than other soil samples. This also correlates with increased organic content (32%), neutral pH (7.2) and bacterial density (2.5×10^9 CFU/g). Generally, sand textured soil and clay portion showed low and high electrical conductivity respectively. This is due to the particle size of soil and nutrient content, which greatly influences bacterial population (EL-Nahhal *et al*., 2014).

6.1.5 Correlation analysis of physicochemical properties with bacterial count

Bacterial growth in different soil samples was correlated with each physical and chemical parameters of soils from different sources in both agricultural field and non-agricultural sites. Among these soil parameters of the agriculture field, both pH and electrical conductivity were negatively correlated with the bacterial count. Total bacterial count was positively correlated with organic carbon content ($r = 0.030$; $P = 0.930$) and negatively correlated with pH ($r = -0.148$; $P= 0.664$) and electrical conductivity($r = -0.375$; $P = 0.256$). Only organic carbon content was correlated with the bacterial count at the significance level (0.01). Among these soil parameters of non-agriculture sites, pH and electrical conductivity were positively correlated with the bacterial count. Total bacterial count was positively correlated with organic carbon content ($r = 0.984$; $P = 0.000$), pH ($r = 0.013$; $P= 0.973$) and electrical conductivity ($r = 0.793$; $P = 0.011$) at (0.05) level of significance. Only organic carbon content was correlated with the bacterial count at the significance level (0.01).

Similarly, bacterial growth in three ecosystem in which the bacterial count was significantly higher in humid ecosystem and was correlated with soil texture such as clay and slity clay ($P<0.005$) and no significant relationship was observed between fungi, actinomycetes and soil physicochemical properties. Bacterial abundance displayed a positive linear relationship ($P = 0.005$) with soil pH and electrical conductivity. It also showed that soil organic matter was higher in the forest ecosystem but lower in agroecosystem soils (Meliani *et al.*, 2012). The importance of soil physicochemical properties influencing soil bacterial communities from 12

locations in Sunderben mangrove forests, Bangladesh showed that soil pH, moisture, nitrogen content varied greatly among the studied locations. The total bacterial colony's enumeration remains higher in Koromjal site ($14.5x10^4$ CFU/g soil) than that in the Hironpoint site ($7.65x10^4$ CFU/g soil).There was no significant correlation among soil physico-chemical properties in the study area. Only soil total nitrogen (%) content showed positive correlation ($r = 0.718$, $P<0.01$) (Hossain *et al.*, 2012).

A study conducted in Megalaya where bacterial count was higher in high altitude forest stand and positively correlated with moisture content ($r = 0.70$; $p<0.05$), organic carbon ($r=0.77$; $p<0.05$) and at low altitude forest stand, it was positively correlated with organic carbon only ($r= 0.70$; $p =0.05$) Laldinthar and Dkhar, (2015). The bacterial population was significantly affected with clay concentration ($r =0.90$), pH ($r=-0.85$), organic matter($r=0.80$), total nitrogen ($r=0.81$) and C/N ratio ($r=0.92$). The organic carbon content significantly ($p<0.05$) correlated with the bacterial population in clay loam and silty clay loams compared to other types of soils (Mohammad, 2015).

6.2 Biochemical characterization of bacterial strains

6.2.1 Bacterial strains from agricultural soil samples

The bacterial strains were isolated and subjected to biochemical tests for identification. Out of 11isolated strains, seven strains (GRS, PAD3, BAN1, BAN2, PAD2, SUG1, and SUG2) were Gram-positive and four were (BAM, PAD1, ANUP and MLK) Gram-negative. All bacterial strains were motile, and positive for catalase,

gelatin hydrolysis, nitrate reduction tests and negative to Indole test, MR test. Results indicated that all are aerobic bacteria due to catalase enzyme, which neutralizes hydrogen peroxide into oxygen and water (Cappucino and Sherman, 1999). These biochemical tests are used to identify bacterial species by differentiating them based on biochemical activities based upon the enzyme production and carbohydrate metabolism.

6.2.2 Bacterial strains from non-agricultural sites

The bacterial strains were isolated and subjected to biochemical tests for identification. Out of nine isolated strains, six strains showed Gram-positive and three strains were Gram- negative. All bacterial strains were motile except a strain isolated from pond sediment (PON1). Only two strains isolated from sewage sediment (SWY and SWV) and one from irrigational canal sediment (CWT2) were positive in oxidase and gelatin hydrolysis tests. These results indicated that most of bacterial strains isolated from both agricultural and non agricultural sites were aerobic and Gram-positive strains, which produce toxins in the extracellular medium.

6.3 Characterization of extracellular proteins from bacterial strains

6.3.1 Total extracellular protein concentration

The extracellular proteins isolated from bacterial strains were estimated by Lowry's method. In this present study, the concentration of extracellular proteins ranged from 10 to 120 µg/ml. The bacterial strain from sewage sediment showed a higher amount of extracellular proteins (120 µg/ml) when compared to rhizosphere

soils collected from agricultural field. There are many reports of varying levels of activities of toxins from *B. thuringiensis* var *israelensis* and *B. sphaericus* on larvae of different mosquito species and these toxins act on the gut wall of susceptible hosts. Some *B. thuringiensis* strains produce nonspecific entomopathogenic exotoxins and are excreated at early vegetative cells that are found in the supernatant fluid after centrifugation of culture fluid (Hernandez *et al*. 2001). Exotoxins of *Pseudomonas* species are also toxic to larvae of mosquitoes and lepidopteran insects. Depending upon the toxin's nature, their action differs among the different species and stages of insects. In particular, exotoxins produced by *Pseudomonas aeruginosa* (Schroeter) Migula reported to be absorbed through the cuticle of insects and act on the haemolymph proteins (Kucera and Lysenko, 1971). Murty *et al.*, (1994) reported that the culture supernatants of a novel *P.fluorescens* strain MSS-1 produced a thermostable, low molecular weight compound effective against *Culex quinquefasciatus*, *Anopheles stephensi and Aedes aegypti.* A formulation was developed from the metabolite of a novel *Pseudomonas fluorescens* Migula strain (VCRC B426) and the lethal fraction was purified from the culture broth, and its molecular mass was determined by high-performance liquid chromatography was 44kDa. This was reported as microbial formulation acting upon mosquito pupae, a non-feeding stage (Prabakaran *et al*, 2003). Similarly, the approximate amount of toxin produced by the cell-free broth of *Pseudomonas fluorescencs* (NCIM-2631) and *Pseudomonas caryophilly* (NCIM-5094) was estimated by the Lowry method in which exotoxin concentration was reported as 4 mg/ml and 3mg/ml respectively.

Among these, *P*. *fluorescencs* (NCIM-2631) showed higher larvicidal activity (Mahamuni *et al*., 2016)

6.3.2 Hemolytic activity of extracellular proteins from bacterial strains

The extracellular proteins were isolated and partially purified using 50% ammonium sulfate precipitation. These exoproteins were tested for toxigenicity by hemolytic assay and the extent of hemolysis was quantified using microhemolytic assay. The variation of hemolysis range from 15% to 71%. In this present study, strain from paddy rhizosphere soil exhibit 71% of hemolytic activity against S-RBC followed by strain from sugarcane field with 62%.The lowest hemolytic activity was observed in bacterial strain from sediment soil with 15%.

Earlier studies showed that *Bacillus thuringiensis* Crystal (Cry) and Cytolytic (Cyt) protein families are a diverse group of proteins with activity against insects of different orders, Lepidoptera, Coleoptera, Diptera and also against other invertebrates such as nematodes. Their primary action is to lyse midgut epithelial cells by inserting them into the target membrane and forming pores (Bravo *et al*., 2007). Notably, *Bt* subspecies *israelensis (Bti)* and *Bt* subspecies *kyushuensis* demonstrated hemolytic activity against horse, sheep and human erythrocytes. The degree of activity varied depending on the bacterial strains and the source of erythrocytes (Ishii and Ohba, 1994). The first recognized mosquitocidal bacterium, *Bacillus thuringiensis* var *israelensis*, crystal Cry and Cyt proteins are involved in the recognition of the insect target, which causes the disruption of the membrane and finally hemolysis. The mode

of action was found that Cry15 has nonspecific pore-forming activity and displays hemolysis on mouse erythrocytes. (Lacey, 2007; Naimov *et al*.,2008).

Vijaypreethi and Pandian, (2008) also reported that the exotoxin of *Pseudomonas fluorescens* Migula was isolated and tested against larval and pupal stages of vector mosquitoes, *Aedes aegypti* (L.) and *Culex quinquefasciatus* Say. The correlation between the concentration of exotoxin with that of the percentage of hemolysis was high (correlation co-efficient (r) value = 0.901) indicating positive and significant correlation between the exotoxin concentrations of the different strains and their haemolytic activity. Similarly, Geetha *et al.,* 2014 also reported a strain of *Bacillus amyloliqufasciens* (VCRC B483) isolated from mangrove forest which produced larvicidal and pupicidal surfactant also exhibited beta hemolysis and bio-film forming capacity. Similar hemolytic pattern was observed in all the nine strains isolated from soil samples in Western Cuba which showed larvicidal activity against *Aedes* sp and *Culex* sp (Gonzalez *et al*., 2013). Therefore, it has been clearly stated that hemolysis is a way to evaluate membrane perturbation which is a commonly required feature present in entomopathogenic toxins (Lopez-Diaz *et al.*, 2013).

6.4 Larvicidal activity of bacterial strains against mosquito vectors

6.4.1 *Aedes aegypti* and *Culex quinquefasciatus*

The mortality rate of larvae for different concentrations of extracellular proteins from bacterial strains of agricultural field and non-agricultural sites were evaluated after 24 hrs and 48 hrs treatment. In 24 hrs treatment, the larvicidal activity

was recorded highest in bacterial strain from paddy field (PAD3) compared to other strains. After 48 hrs treatment, the mortality percentage of two larvae by bacterial strain (PAD3) increased with increased exposure time. But in non-agricultural sites, none of the bacterial strain showed a significant mortality rate than bacterial strain from the agricultural field. In the present study, two mosquito species, *Aedes aegypti* and *Culex quinquefasciatus* were tested with culture filtrate containing exoproteins, *Aedes aegypti* was more resistant when compared to *Cx. quinquefasciatus*. The LC_{50} and LC_{90} values of *Ae. aegypti* and *Cx. quinquefasciatus* were 15.193 (14.018-16.306) µg/ml, 21.277 (19.763-588.297) µg/ml and 13.268(12.032-14.359) µg/ml, 18.975(17.528-21.243) µg/ml respectively. The results showed that the larval mortality of *Cx. quinquefasciatus* occurred with a lower concentration of exoproteins when compared with *Ae. aegypti*.

Similarly, El-Kersh *et al*., (2016) reported that eight larvicidal bacterial strains were isolated from soil showed activity (LC_{50} = (3.90-7.40 µg/ml) against *Anopheles gambiae* upto 3.4 fold higher than the reference Bti-H14 strain (LC_{50}=13.33µg/ml). The mortality rate of larvae is also determined by the concentration of exoproteins in the culture filtrate and depends on the vector species. It was clearly shown that *An. stephensi* were the most vulnerable with LC_{50} of 5 µg/ ml. In contrast, *Cx. quinquefasciatus* and *Ae. aegypti* pupae required 8.1 and 13.2 µg/ml, which were 1.62 and 2.64 times higher than that required for *An. Stephensi* (Geetha *et al*., 2014).

Prabakaran *et al*., (2003) also reported the lethal concentration of exoproteins of *P.fluorescens* against mosquito vectors, *Ae.aegypti*, *An. stephensi* and *Cx.*

quinquefasciatus. Among the three species of mosquitoes, *An. stephensi* remain as most susceptible, whereas *Ae. aegypti* was the least susceptible. Balakrishnan *et al.*, (2015) also reported that the culture supernatants of *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus sphaericus* and *Bacillus cereus* at different concentrations were tested against third instar larvae and pupae of *An. stephensi* and *Ae. aegypti*. The culture supernatant of *B. thuringiensis* (5µl/ml) was toxic against pupae of *An. stephensi* and *Ae. aegypti*.

Bukhari and Shakoori, 2010 also reported that *B.t* spore remains effective against third instar larva of *An.stephensi*. Results showed that SBS *Bt*-45 showed 100% mortality at 800µg/spores/ml. The LC_{50} value of Bt strain SBS *Bt*-45 isolated from cattle waste indicated as 366 $\pm$ 0.7 µg/ml. Nabar and Lokegaonkar, 2015 reported that out of 24 bacterial isolates screened, 21 bacterial isolates were found effective against larvae of *Aedes* and *Culex* species. The secondary metabolites of *Bacillus* and *Pseudomonas* were evaluated for toxicity, which showed 20% of secondary metabolites showed 100% mortality against *Culex* sp at 200ppm, 50% secondary metabolites of thermophilic bacterial isolates remain low as 150 ppm.

Similarly, Lalithambika, 2014 also reported that larvicidal activity *P. fluorescens* from the rhizosphere soil, the anti-larvicidal activity was checked against *Ae. aegypti* larvae using liquid formulation of the exoproteins. Kings B yeast extract medium showed 100% mortality for 24 hrs supernatant at a protein concentration of 80µg/ml and at 40µg/ml and 80µg/ml for 48 hrs supernatant. This showed that mosquito larvae's net mortality rate of increases with exposure time to exoproteins.

Padmanabhan *et al*, (2005) also reported that the formulation prepared from the exotoxin of *P. fluorescens* was toxic to the house fly. Pupae of housefly were more susceptible than larvae and the activity of the toxin might have been through cuticular absorption. The LC_{50} and LC_{90} values calculated from the net mortality of larvae and pupae together were 8.25 and 51.79 μg protein/g respectively, from day 1 to 12 post-treatment.

Shukla *et al.,* 2017 also reported that the isolated Pseudomonas aeruginosa showed larvicidal bioassay significant LC50 and LC90 values (5.58 mg/Ml) and (12.80 mg/Ml) against fourth instar larvae of *Aedes aegypti*. The effect of toxic compounds present in extracellular metabolites of bacteria entered the larvae through cuticle and causes distruption.

Ruiu *et al*, (2017) reported that bacterial strains isolated from septicaemic *Galleria mellonella* larvae reported as new entomopathogenic bacterial strains such as *Alcaligenes aquatilis, Alcaligenes faecalis, Enterococcus mundtii, Pseudomonas protogens, Serratia nematodiphilia, Serratia marcescens and Stenotrophomonas maltophila* in which the filtered culture supernatants of these strains induce oral insecticidal activity in test population compared to reference strains.

6.4.2 Regression analysis of larval mortality rate by bacterial strains

Regression analysis showed that the mosquito larval mortality rate (Y) of *Aedes aegypti* was significantly positively correlated with the concentration of bacterial exoproteins of agricultural field soil samples with respect to exposure period

($p<0.05$) and the regression equation and coefficient values were calculated for PAD3 (y =-33.19+5.49,R^2 =0.975), SUG2 (y= 27.90+2.57, R^2 =0.921) and PAD1 (y=-24.56+1.82, R^2 =0.988) in fig.5.10. Similarly, the regression equation and coefficient values were calculated as PAD3(y=-26.69+5.79, R^2 =0.980), SUG2 (y= -21.74+2.669, R^2 =0.995) and PAD1 (y=-9.50+1.43, R^2 =0.990) respectively (Fig 5.12). The results of the regression analysis revealed that the mosquito larval mortality rate (Y) was significantly positively correlated with the concentration of bacterial exoproteins with respect to exposure period ($p<0.05$) In this present study, out of twenty bacterial strains screened against third instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus*, the above three bacterial strains were highly effective in causing mortality of larvae at a minimum concentration of exoproteins. The R^2 value indicates how much of the total variation in the dependent variable. The p-value indicates whether this relationship between two variables is significant or not.

Similarly, a study was conducted to determine mortality rate of third instar larvae of *Aedes aegypti* at 24 hrs post- treatment with toxic extract of *Pseudomonas frederiksbergiensis* at concentrations ranging from 3.5- 1000µl/l and above 500µl/ml showed 100% mortality. A linear relationship between concentration and mean mortality percentages was analysed using regression plot. (M = -4.08333 + 0.2130C) and indicated the increase in the mortality rate with the concentration of toxic extract of bacterial strain (Ahmed *et al*.,2014). The regression equation values of *B. subtilis* for third instar larvae or pupa of *A. stephensi* were Y = -2.094+ 0.550 X and Y = -1.946 + 0.616 X while for *A. aegypti* Y = -2.051 + 0.548 X and Y = -1.930 +

0.591 X, respectively. Among the third instar larvae were more susceptible than the pupae. The B. *subtilis* showed considerable larvicidal and pupicidal activity. These values were more significant (P < 0.05) for *Ae. aegypti* than *An. stephensi* (Balakrishnan *et al.,* 2015).

6.5 Molecular characterization of bacterial strains

The larvicidal activity of twenty bacterial strains from both agricultural and non-agricultural sites was screened against *Ae. aegypti* and *Cx. quinquefasciatus* which showed three bacterial strains found effective against these species. Molecular characterization of these bacterial strains was done and identified as *Bacillus cereus*, *Bacillus firmus* and *Pseudomonas aeruginosa*. Similarly, Poopathi *et al*, 2014 reported that the preliminary screening of 1000 bacterial isolates from excreta of wild birds resulted in twelve isolates, which were found effective against *Aedes*, *Culex* and *Anopheles* species. These bacterial isolates were identified as *Bacillus thuringiensis*, *B.sphaericus* and *B.cereus*. Molecular characterization showed these effective strains *Bacillus cereus,* and *Bacillus thuringiensis* showed similarly to *Bti.*

Lukenge *et al*, 2017 reported that out of 616 samples examined from tree hole debris in Uganda, 21 strains were active against *Aedes aegypti* and *Anopheles gambiae*. Phylogenetic analysis revealed that isolates MR-01, 04, 05 related to *Bacillus thuringiensis serovar Kurstaki* strain YBT-1520 by 100% homology and isolates MR-06 and 07 revealed 99% homology to *Bacillus thuringiensis* YBT-1518 and *Bacillus thuringiensis* strain Lr 7/2 respectively. Isolates MR-12 and MR-21

showed 97% homology with *Bacillus anthracis* strain Shikan-NIID. All the strains were Gram- positive, endospore forming which showed 97-100% homology with *Bacillus* spp.

6.5.1 *Bacillus cereus*

Bacillus cereus were isolated from paddy rhizosphere soil, sugarcane rhizosphere soil and sewage sediment soil. Larvicidal activity studies reported that *Bacillus cereus* strain isolated from paddy rhizosphere showed highest mortality rate of *Ae. aegypti* and *Cx. quinquefasciatus* at minimum concentration of (LC_{50}-15.193µg/ml) and (LC_{50}-13.268µg/ml) respectively than other two strains isolated from sugarcane rhizosphere (LC_{50}-42.609µg/ml) and (LC_{50}-33.232µg/ml), strain isolated from sewage sediment (LC_{50}-249.270µg/ml) and (LC_{50}-231.359µg/ml). *B. cereus is* a Gram-positive, spore-forming rod shaped bacteria widely distributed in the environment. They are reported in the excreta of birds, marine environment (Mani *et al.*, 2018) and also in the guts of mosquito larvae (Pleampis *et al*., 2001). Similar observations have also been reported from *B. cereus* (M413, C32, Ts-25, VB-17, VB-24) isolated from diverse the environment such as dead mosquito larvae, sediments, ditches and clinical samples. It acts as a natural facultative mosquito pathogen (Chaterjee *et al*., 2010). They also encode a range of toxins and other extracellular virulence factors on the chromosome and these genes are similar to *B. thuringiensis* strains, which are meant for mosquitocidal activity (Maughan and Van der Auwera, 2011).In addition, *B. cereus* strains produces haemolysins and phospholipases that are probably essential virulence determinants. The thiol-activated cytolysins of both

B. thuringiensis var. *kurstaki* and *B. cereus* have been purified and shown to be biologically, physiochemically and immunologically identical (Honda et *al.* 1991)

6.5.2 *Bacillus firmus*

In this study, *Bacillus firmus* strain was isolated from sugarcane rhizosphere soil which showed mosquitocidal activity against *Ae. aegypti* and *Cx. quinquefasciatus* vectors at concentration of (LC_{50}-30.817µg/ml) and (LC_{50}-27.221µg/ml) respectively. Geng *et al.,* 2016 reported that *B. firmus* strain DS-1 has high toxicity against *Meloidogyne incognita* and soybean cyst nematode. It includes many virulence factors incuding peptidase S8 superfamily protein called Sep1 exhibited serine protease activity and degraded the intestinal tissues of nematodes. Thus, the Sep1 protease of *B. firmus* is a novel biocontrol factor with activity against a root-knot nematode.

6.5.3 *Pseudomonas aeruginosa*

Pseudomonas species have been known to exhibit cytotoxicity against lepidopteran insects and mosquito larvae Migula, an exotoxin released by *P. aeruginosa* acts upon hemolymph proteins of insects (Kucera and Lysenko, 1971. Lalithambika and Vani, 2016 also reported that the protein concentrations in the culture supernatant of the *P. aeruginosa* (KUN2) showed 100% mortality against *Ae. aegypti* larvae in the 24 hrs of exposure at 100 and 150 µg concentration. The exotoxins of microbial origin, including *Pseudomonas* species, have been known to be toxic to the larvae of mosquitoes as well as lepidopterans. Prabakaran *et al.,*(2009)

also reported (GPS medium) of *P. fluorescens* showed maximum larvicidal activity against *Ae. aegypti* larvae when compared to the larvae of *An. stephensi* and *Cx. quinquefasciatus.*

6.5.4 *Cupriavidus taiwanensis*

In the present study, *Cupriavidus taiwanensis* isolated from Bamboo rhizosphere showed mosquitocidal activity at (LC_{50}-264.073µg/ml) and (LC_{50}-206.804µg/ml). *Cupriavidus taiwanensis* (originally called Ralstonia taiwanensis or Wautersia taiwanensis), a betaproteobacteria of the rhizobia group, was isolated from a nodule from the legume species Mimosa pudica in Taiwan. It has only recently been discovered that in addition to alphaproteobacteria some betaproteobacteria can nodulate legumes and fix atmospheric nitrogen. They have been isolated from soil, water, plants, rhizosphere, insects (Burkholderia), and from infected humans (Tran Van *et al.,* 2000)

6.5.5 *Acinetobacter baumanii*

In this present study, *Acinetobacter baumannii* was isolated from pond sediment showed mosquitocidal activity at (LC_{50}-321.387µg/ml) and (LC_{50}-317.710 µg/ml) against *Ae. agypti* and *Cx. quinquefasciatus* respectively. It is an obligate aerobic bacterium commonly found in soil, water and sewage, as well as in hospital environments (Smith et al., 2007b). *Acinetobactor sp.* is common soil borne coccoid bacteria that also give 97 ± 5% mortality rates. It was also reported that *Acinetobactor sp.* active against summer cockchafer, *Melolontha melolontha* (Coleoptera:

Scarabaeidae). Furthermore, *Acinetobactor spp.* can be potent for the control of mosquito larvae (Smani *et al.*,2012).

6.5.6 *Enterobacter hormaechei*

In this present study, *Enterobacter hormaechei* isolated from irrigational sediment soil showed mosquitocidal activity against *Ae.aegypti* (LC_{50}-265.546µg/ml) and *Cx. quinquefasciatus* (LC_{50}-265.656µg/ml).*Enterobacter hormaechei* is a species of gram-negative bacterium, belonging to the *Enterobacter cloacae complex* (ECC), which can exist in Mangrove sediments and also in the intestinal tract of humans and animals (Balakrishnan *et al*., 2019)

6.5.7 *Exiguobacterium profundum*

Exiguobacterium profundum isolated from mosquito breeding site showed mosquitocidal activity against *Aedes aegypti* (LC_{50}-326.040µg/ml) and *Culex quinquefasciatus* (LC_{50}-205.595µg/ml). Members of the genus *Exiguobacterium* are found in diverse environments from marine, freshwaters to hot springs. They can grow in a wide range of temperature, pH, salinity, and heavy-metal concentrations include sewage sediments (Crapart *et al*., 2007).

6.6 Morphological changes in treated mosquito larvae with exoproteins from *Bacillus cereus*

Both control and experiment larvae of *Ae. aegypti* and *Cx. quinquefasciatus* of 24 hrs post-treatment with exoproteins of highly effective strain *Bacillus cereus* were observed under Scanning Electron microscope. The SEM pictures indicated that the

control larvae remain intact without any deformations but treated larva with bacterial exotoxin showed apparent shrinkage of the body parts. Particularly, the thorax and abdomen regions were completely distorted, but the head region remained intact without any physiological changes

Similarly**,** both control and *P. frederiksbergensis* extract-treated larvae of *Culex pipens* were observed under scanning Electron microscope showed a significant shrinkage in the body, particularly in the thorax region. The thorax showed a significant 46.8% reduction in size compared to the control. But head capsule was similar in size for both infected and control larvae without any shrinking (Ahmed *et al.*, 2014). The histopathological study of *Penicillium daleae* ethylacetate extract treated with *Ae. aegypti* and *Cx. quinquefasciatus* larvae showed collapse and broken epithelial cell layer. In contrast, target mosquitoes showed entire damage in the midgut and caeca areas, and finally, larval structures were fully collapsed. Moreover, a huge shrinkage was observed in the abdominal region of *Aedes aegypti* and *Culex quinquefasciatus* treated with mycelia ethylacetate extract (Ragavendran *et al*., 2017).

Summary

7.0 Summary

Mosquito borne diseases are prevalent in many countries affecting over 700 million people globally and 40 million of the Indian population. As a vector, different species of mosquitoes are capable of transmitting life-threatening diseases like malaria, yellow fever, dengue, chikungunya, filariasis, encephalitis *etc.* The prevalence of mosquito borne diseases are recorded in almost all tropical and subtropical countries and also temperate countries recently.

Generally, chemical pesticides are commonly used to eradicate vector populations, but pesticides also targets non-vector insect populations. Frequent use of pesticides causes deposition of pesticide residues in the environment which leads to bio-magnification. Besides, resistance to commonly used pesticides limits the use of pesticides in mosquito control programs. To overcome this, the use of biocontrol agents remains as the best option for killing the pest. Hence, it is necessary to identify a safe, eco-friendly and efficient larvicide to control mosquito menace. At present, microbial insecticides are considered the main component of the bio-pesticide industry. Hence, extensive research began to explore new microbes with useful metabolites that can serve as alternative source of mosquito control agents.

The study was proposed to evaluate the bioefficacy of microbes from soil samples collected from Madurai District, Tamil Nadu, India. The sampling sites were selected which include both agricultural fields and non-agricultural areas. Agricultural field samples were collected from the rhizosphere of paddy, banana, sugarcane,

bamboo, Animal fodder farm soil, vegetable farm soil and garden soil. Non-agricultural sites include pond sediment soil, sewage sediment, mosquito breeding site soil and irrigation side by soil. The physico-chemical properties of soil samples such as pH, electrical conductivity, soil texture and organic carbon content were analyzed by standard procedures. Pure culture of bacterial strains were isolated and named with reference codes. Among 11 sampling sites of agricultural field samples, paddy rhizosphere soil samples showed the highest bacterial count. The higher microbial population could be correlatd with higher organic carbon content, soil texture (slit and clay) greatly influence higher bacterial growth.

Among the nine non-agricultural site samples, only sewage sediment reported higher bacterial growth due to presence of organic substrates in sewage sediment samples. The above results showed that bacterial growth in both agricultural and non-agricultural samples showed variations in physico-chemical properties of soil samples. The above results were checked and confirmed by the statistical analysis, which showed a significant correlation between bacterial count and soil physico-chemical characteristics. Only organic carbon content was correlated with the bacterial count at a significant level ($p<0.01$). This showed that bacterial growth in the agricultural area was greater with organic carbon content than soil pH, soil texture, and electrical conductivity. Similar results were also observed in non-agricultural site samples.

To isolate exoproteins from bacterial strains, bacterial culture filtrates were subjected to partial purification. Among these, a strain isolated from pond sediment

showed a higher concentration of exoprotein as 120 ± 2.4 µg/ml. To determine the pathogenicity of isolated bacterial strains, they were subjected to hemolytic activity against 1% sheep red blood cells. Among all bacterial strains, only seven bacterial strains exhibited more than 50% hemolytic activity whereas nine bacterial strains showed a moderate hemolytic activity between 25 to 50%. Other bacterial strains showed a poor or mild hemolytic activity of less than 25%. As hemolytic property acts as desired characteristics of entomopathogenic strains, isolated PAD3 strain effectively lyses 1% red blood cells, which cause membrane disturbance in the insects and act as potential mosquitocidal strain.

World Health Organization Pesticides Evaluation (WHOPES) has given certain guidelines to test bacterial larvicides by determining lethal concentration of exoproteins, which cause 50% and 90% of larval mortality rate. In this study, exoproteins of all isolated bacterial strains were tested against third instar larvae of *Aedes aegypti*, a mosquito vector of dengue and *Culex quinquefasciatus*, a mosquito vector of filarisis. After 24 hrs and 48 h treatment with exoproteins, the larvicidal activity was observed. The highest larvicidal activity was observed in a strain isolated from paddy rhizosphere soil (PAD3) followed by a strain isolated from sugarcane rhizosphere soil (SUG2) and a strain from another paddy rhizosphere soil (PAD1). The mortality rates of *Aedes aegypti* and *Culex quinquefasciatus* larva were proportionately increased with increased exposure time.

Similarly, all isolates from non-agricultural site samples, all isolated bacterial strains were tested against third instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus.*

After 24 hrs and 48 hrs treatment with exoproteins of bacterial strains, the strain PON2 isolated from pond sediment showed highest larvicidal activity among the isolates from non-agricultural sites. Overall, the strain PAD3 strain showed the highest mortality with an LC_{50} value of 15.19 µg/ml than the strain PON2 with an LC_{50} of 232.097µg/ml.

Regression analysis showed that *Ae. aegypti* and *Cx. quinquefasciatus* larval mortality rate (Y) was significantly increased with the concentration of *B. cereus* strain exoproteins (X) with respective exposure period at a significant level ($p<0.05$). The above studies indicated that *B. cereus* strain effectively damaged the abdominal region of larvae and could act as a gut poison. Further, *Bacillus cereus* proteins will be characterized and formulated with suitable carrier materials. The formulated products will be tested in the field to further evaluate the bioefficacy against mosquito larva in the breeding grounds.

Molecular characterization of bacterial strains showed that effective mosquitocidal bacterial strains were PAD3, SUG2, and PAD1 as *Bacillus cereus*, *Bacillus firmus*, and *Pseudomonas aeruginosa* respectively. Scanning Electron Microscopic analysis was done to identify the morphological changes in the body parts of both *Ae. aegypti* and *Cx. quinquefasciatus* larvae. The *Ae.aegypti* larvae treated with exoproteins of *B. cereus* showed morphological changes such as disintegration of the body except the head region. Similarly, *Cx. quinquefasciatus* larva treated with *B. cereus* exoproteins showed clear shrinkage of the body parts except head region.

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List of Publications

PAPER PUBLICATIONS

- **Sivakami, V.**, Kannan, S. and Selvaraj Pandian, R., 2018. SELECTION OF SUITABLE BACTERIAL ISOLATES FOR THEIR USAGE AS BIOCONTROL AGENTS AGAINST MEDICALLY IMPORTANT PESTS, *International Journal of Recent Scientific Research*, 9(2), 24521-24524.

- **Sivakami, V**., Kannan, S., Rajendhran, J. and Selvaraj Pandian, R., 2017. Biocontrol efficacy of selected mosquitocidal bacteria. *International Journal of Life Sciences*, 5(4), 577-586.

PAPER PRESENTED

- Presented a research paper as poster entitled, **"Biolarvicidal activity of bacterial strains against mosquitoes with reference to their toxigenicity"** in 49th Aqua-Terr Annual Conference on Biological Sciences conducted by School of Biological Sciences, Madurai Kamaraj University, Madurai on 27-28 February, 2018.

- Presented a research paper as poster entitled '**Biocontrol efficacy of selected soil microbes'** in the ENERGY FEST'17 Seminar on Advanced Technologies and Innovations in Energy and Environment organized by School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai on 28th March, 2017.

- Participated and presented a poster entitled '**Mosquitocidal efficacy of selected soil microbes'** in International conference on Advanced Functional Materials for Energy, Environment and Biomedical Applications (AFMEED-2017) held at Madurai Kamaraj University on 11 & 12 December, 2017.

- Presented a research paper as poster entitled, '**Microbes in the sustainable Environment'** in the 9th NABS National Conference on New Biological Researches: Opportunities and Challenges for Sustainable Development organized by School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai on 11th & 12th August, 2016.

- Presented a research paper as poster entitled,' **Enhancement of food mediated immunity in consumers by a bacterium isolated from soil**' in the International conference on New Horizons, Emerging Challenges and Recent Paradigms in Food Mediated Immunity and their role in Human health and Longevity organized by PG Department of Zoology and Department of Immunology and Microbiology, The American College, Madurai on 31st August to 1st September, 2016.

SEMINARS AND WORKSHOPS PARTICIPATED:

- Participated in two days **workshop on Nanomaterials characterization by Electron Microscopy (WNCEM-2018)** conducted by Madurai Kamaraj University on 21st & 22nd February, 2018.
- Participated in Science Academies Lecture **Workshop on Introduction to Modern Biology** held in The American College, Madurai on 19th to 21st January, 2017.
- Participated in one day **National seminar on Recent Trends in Ecotoxicological Research** conducted by Department of Zoology, Directorate of Distance Education, Madurai Kamaraj University on 9th August, 2017.
- Participated in one day **workshop and awareness programme on Solar Rooftop segments** organized by Department of Solar Energy, School of Energy Sciences, Madurai Kamaraj University, Madurai on 30th May, 2016.
- Participated in one day **workshop on Curriculum Development on Nanoscience and Nanotechnology** (CDNSNT-16) conducted by Nanosciences and Technology, Curriculum Development Cell (CDC), Madurai Kamaraj University on 7th October, 2016.



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Research Article

SELECTION OF SUITABLE BACTERIAL ISOLATES FOR THEIR USAGE AS BIOCONTROL AGENTS AGAINST MEDICALLY IMPORTANT PESTS

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ABSTRACT

Microbial populations are highly diversified in nature due to their metabolic diversity and genetic adaptability which enable them to survive in different ecological niches. The rhizosphere region of a specific plant inhabits specific microflora which produces numerous compounds involved in complex mechanisms such as biofertilization, phytostimulation and biocontrol activity. Among this, *Pseudomonas* genera plays a major role in biocontrol mechanisms due to their diverse metabolic compounds. These compounds are exploited as biocontrol agents against medically important vectors such as mosquitoes which pose a major threat to public health. Hence it is essential for identifying a potential candidate as alternative to chemical insecticides. In this context, the present study was conducted to isolate *Pseudomonas fluorescens* strains from different sources. Their toxigenicity were assayed and evaluated for biocontrol efficacy against medically important mosquito vector, *Aedes aegypti* (Linn).These effective strains proved to be potential candidate for bioinsecticide formulation and field application trails.

INTRODUCTION

Microbial community in the plant rhizosphere soil is highly dynamic in nature which compete for water, nutrients, space and also help in the growth and ecological fitness of their host. The diversity and predominance of microbial population depend on a number of abiotic and biotic factors of a particular ecological niche. Plant species, plant development stage and soil type have been indicated as major factors determining the composition of rhizosphere microbial communities (Broeckling *et al*, 2008). The rhizosphere microflora include bacteria, fungi, nematodes, protozoa, algae and microarthops (Raaijmaker *et al*, 2009). Among these, *Pseudomonas* spp and *Bacillus* spp are well documented bacterial populations which involve in biostimulation, biofertilization and biocontrol activities. Thus, rhizosphere zone acts as bioresource for bioactive substances such as antibiotics, biosurfactants, enzymes and osmoprotective substances (Berg *et al*, 2005).

Species of the genus *Pseudomonas* show remarkable metabolic and physiological versality, enabling colonization of diverse terrestrial and aquatic habitats (Palleroni,1992) and produce a variety of metabolites which posses a major role in biocontrol of phytophathogens and mosquitoes (Haas and Defago, 2005). *P.fluorescens* Migula (VCRC-B426) culture filtrate was found to be effective against pupae of mosquitoes such as *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* (Prabakaran *et al*, 2003, Padmanaban *et al*, 2005). This strain plays a significant role in the degradation of cuticular regions and peritrophic membrane and binds to the midgut epithelium of larvae and pupae of mosquito species of *C. quinquefasciatus* (Usharani & Kummankottil, 2012). The secondary metabolite or exotoxin produced by *Pseudomonas fluorescens* was found to be a Rhamnolipid which act as biosurfactant (Prabakaran *et al*, 2015). Hence, an attempt was made to isolate novel strains from native environment which can be effectively used for field applications. In this context, the present study was conducted to isolate *Pseudomonas fluorescens* strains from both rhizosphere regions and non rhizosphere regions and to evaluate the biocontrol efficacy against dengue vector, *Aedes aegypti*.

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MATERIALS AND METHODS

Study site description and soil sampling

In the present study, a total of six soil samples were collected from different habitats such as such as paddy field, bamboo tree soil and garden soil and sewage effluent discharging sites. About 10 cm rhizosphere soil particles loosely adhering to the roots were gently teased out and the soil was aspetically transferred to sterile polythene bags. In addition, sewage sediment samples were collected from mosquito breeding sites. All the soil samples were added to a conical flask and shaken with 100 ml sterile distilled water to obtain standard soil suspension. This will enrich the growth of soil microbes. From this, soil suspension was serially diluted and plated on selective medium Pseudomonas Agar (King *et al*, 1954) and it was obtained from Hi Media Pvt. Ltd.

Identification of Pseudomonas spp from samples

All the isolates were subjected to biochemical characterization according to Bergey's Manual of Determinative Bacteriology. Biochemical tests such as Nitrate test, HCN production, IAA test, starch hydrolysis, gelatin liquefaction were done to the identify all the cultured isolates to species level (Holt *et al*, 1994). The isolated and identified strains were given reference codes as PX01, PX02, PX03, PY04, PY05 and PY06 respectively to specify the soil type and the type of ecological niches.

Production of extracellular proteins

Bacterial isolates were inoculated into Glucose Peptone Broth (GPS), enrichment media for the isolation of extracellular proteins. For this, one loopful of slant culture was inoculated into 10ml of GPS medium in a test tube and kept on a rotary shaker at 180 rev min_1 for 7h.

The culture was then transferred to 50ml of GPS medium and the flask was incubated on a rotary shaker at 30°C and 180 rev min_1 for 48h. The cell mass was harvested by centrifugation at 10000 rev min_1 for 15min and the cell-free supernatant was transferred to fresh sterile tubes for further studies (Prabakaran *et al*,2003). The protein concentration of extracellular proteins were determined using Lowry's method.

Hemolytic activity

Hemolytic activity was determined by incubating suspensions of sheep red blood cells with serial dilutions of each sample. Sheep blood was collected from slaughter house with anticoagulant EDTA (2.7g/100ml) and 1% S-RBC was prepared by centrifuging anticoagulated blood with PBS for thrice. Microhemolytic assay was performed using V bottom 96 well microtitre plate. Serial dilution of samples was done with PBS in different rows of microtitre plate.50 µl or S-RBCwas added in all the wells and incubated at room temperature for 1 hr. PBS was used as negative control and 10% Triton-X was used as positive control (Rodriguez *et al*,2014). Uniform red color suspension in the wells was considered as positive hemolysis and a button formation in the bottom of wells was considered as negative hemolysis. The hemolysis was calculated as OD values in Spectrophotometer.

Biocontrol efficacy

The exoproteins in culture supernatant of *Pseudomonas fluorescens* isolates were determined. The test solution for bioassay was prepared by diluting exoproteins with sterile distilled water. *Aedes aegypti*, mosquito larvae procured from CRME (Centre for Research in Medical Entamology), Madurai were used in this study. Larval food containing yeast and dog biscuit (1:1/w:w) was added in fine powder form to the bioassay cups (WHO, 2005). For every 25 ml of test solution, 10 larvae were transferred and a controlset along with three experimental sets were maintained. The mortality rate of larvae was observed after 24hrs. Using Abbott's formula, mortality rate was calculated and probit regression analysis was carried out using SPSS (statistical software package).

RESULTS

All bacterial isolates produced yellowish green colored colonies which showed fluorescence under UV light (λ =356 nm). Microscopic examination revealed Gram negative rods and also positive for motility test, Catalase test, nitrate test, Oxidase test, gelatin hydrolysis.

The extracellular proteins of *Pseudomonas fluorescens* were characterized for protein estimation. These proteins were further partially purified using 50% ammonium sulfate precipitation. Hemolytic activity with 1% S-RBC showed different hemolytic pattern with respective to their sources (fig.1). Biocontrol efficacy of exoproteins against mosquito vector, *Aedes aegypti* showed that LC_{50} values of PX01, PX02, PX03, PY04, PY05, PY06 are 144.8, 29.9, 19.2, 25.3, 38.6, 106.9 µg protein per ml and LC_{90} values are 214, 35.5, 28.6, 43.3, 47, 155.9 µg protein per ml respectively, indicating a diversity in the biological activity.

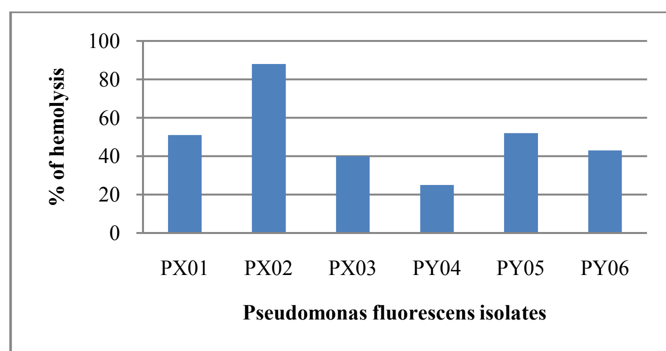


Fig 1 Hemolytic activity of *Pseudomonas fluorscens* isolates from different sources.

X- rhizosphere region, Y- non-rhizosphere region
(PX01-Garden soil, PX02-Bamboo rhizosphere soil, PX03-Paddy rhizosphere soil, PY04-Sewage sediment I,PY05-Sewage sediment II, PY06-Sewage sediment III)

DISCUSSION

Within the rhizosphere soil samples, fluorescent pseudomonads show different characteristics with respect to toxigenicity and biocidal activity. This is mainly due to ecological differences such as soil type, water availability, type of plant species etc. Earlier studies showed that there is a lack of diversity in sandy soils that are frequently tilled (Bronstad *et al*, 1996) and low microbial diversity could be associated with greater selective pressure than organic rich agricultural soils. Bamboo

rhizosphere soil remains as sandy soil with less moisture when compared to paddy rhizosphere and garden soils. Apart from physiochemical complexity of soil type, plant species also determines predominant microflora around their root region which in turn act against phytopathogens, stimulate plant growth and improve soil quality (Grayston *et al*, 1998).

In comparison with non-rhizosphere sampling sites such as sewage sediments, there is a chance of organic pollution and environmental stress which will reduce bacterial diversity. High organic substrates favours the growth of few fast growing bacterial population (Trosvik *et al*, 1996). Despite of these contributing factors, fluorescent pseudomonads with their high genetic adaptability and metabolic versality capable of growing in diverse ecological niches. The extent of hemolysis was quantified using microhemolytic assay and the variation in hemolytic ability of exoproteins of *Pseudomonas fluorescens* strains ranges from 25% to 88%. Similar results were also documented in earlier reports showed that they are highly diversified in nature with reference to their sources (Pushpanathan and Pandian RS, 2008; Vijaypreethi and Pandian RS, 2009). Some strains of non-spore forming bacteria are also toxic to larval stages of mosquitoes as well as lepidopteran insects. *Pseudomonas aeuroginosa* (Migula) produces exotoxin which have been noted to be absorbed through the cuticle of insects and act on the heamolymph proteins (Kucera & Lysenko, 1971). In this present study, extracellular proteins of *Pseudomonas fluoresecens* isolates were analysed for larvicidal effect against dengue vector, *Aedes aegypti* (Linn). The late 3rd instar larvae of this vector remain highly susceptible to exoprotein of isolate PX03 (paddy rhizosphere soil) and are less susceptible to exoproteins of isolate PX01 (garden soil). This showed that *Pseudomonas fluorescens* strains are potential agents to assess the larvicidal activity.

CONCLUSION

In this preliminary study, all the isolates of *Pseudomonas fluorescens* remain effective against *Aedes aegypti* larvae. But few strains from rhizosphere region showed the highest mortality in minimum concentration of exoproteins. Therefore, these strains could be selected as suitable candidate for biocontrol efficacy of mosquito vectors. Further, identification of the toxic compound responsible for the larvicidal activity and molecular characterization has to be done to develop suitable effective strains. In this connection, the present work is in progress.

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REVIEW ARTICLE

Biocontrol efficacy of selected mosquitocidal bacteria

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ABSTRACT

Insect species represent the largest percentage of the world's known species. They are undoubtedly the most adaptable life forms existing on the Earth. Less than 0.5 percentage of the total number of the known insect species are considered as pests. Among these, mosquitoes pose a major threat to public health by transmitting diseases like malaria, dengue, chikungunya etc. Only four classes of chemical insecticides have been approved by WHO with less target sites. Indiscriminate use of these synthetic insecticides and resultant selection pressure on insect populations has caused many mosquito species remain resistant to widely used insecticides. Hence, alternative approach has been initiated to use biological agents. This biocontrol strategy is important in order to counter the evolution of resistance in target populations and possible effects on non target organisms. Unlike chemical insecticides, biocontrol agents are host specific, safer to the environment, find easy application in the field, long-lasting effect with single application and cost effective production. In this context, this paper reviews about the important mosquitocidal bacteria and their efficacy against different mosquito species.

Keywords: Mosquitocidal, Insecticides, resistance, biocontrol agents

INTRODUCTION

Mosquitoes are considered as large group of insects present throughout the temperate and tropical regions and even beyond the Arctic Circle of the world (Harbach, 2007). India is ranked fifth in terms of mosquito biodiversity after Brazil, Indonesia, Malaysia and Thailand (Foley *et al.,* 2007). They belong to family Culicidae, order Diptera and are divided into two subfamilies and 112 genera. At present, a total of 3,540 recognized mosquito species are recorded in the world. Among this, the Indian mosquito fauna includes 393 species which is divided among 49 genera and 41 subgenera. Most of the important disease vectors are the members of Anopheliae and Culicidae. In India, 31 species are currently recognized for

transmitting various mosquito-borne pathogens (Bhattacharya *et al.,* 2014). They act as vectors to transmit most of emerging diseases such as malaria, yellow fever, dengue, chikungunya, filariasis, encephalitis, West Nile fever etc.

Vector borne diseases-Global burden

Vector-borne diseases are responsible for 17% of the global burden of parasitic and infectious diseases (WHO, 2008). Within the past two decades, many important vector-borne diseases have re-emerged or spread to new parts of the world. Traditionally, it is regarded as a problem of tropical countries; now pose an increasingly wider threat to global public health, both in terms of the number of people affected and their geographical spread (WHO, 2014). For example, some of the vector borne diseases such as dengue, chikunguniya and West Nile virus are emerging in the countries where they are previously unknown. This is mainly due to seasonal weather variation, socio-economic status, vector control programmes, environmental changes and drug resistance which are highly like to influence current vector-borne disease epidemiology. These effects are likely to express in many ways from short term epidemics to long-term gradual changes in disease trends (Githeko *et al.,* 2000).

Vector control strategies:

The control of mosquito borne diseases remain a major problem due to the absence of effective vaccines or specific anti-viral drugs. Vector control is a powerful preventive tool that is not used to its full potential. It is defined as measures of any kind, directed against vectors of diseases and intended to limit their ability to transmit diseases (Karunamoorti, 2013). Mosquito control is carried out mainly with chemical insecticides such as organophosphates, carbamates and pyrethroids. Synthetic chemical insecticides such as DDT, delmethrin, malathion, chloropyriphos, etc are popularly used as first line of defense against pest populations particularly mosquito vectors. In 1955, WHO proposed the eradication of most of the prevalent vector-borne disease Malaria with the use of residual house-spraying of DDT (Hemingway and Ranson, 2000). The utilization of these products has been limited because they are non-specific, pollute the environment and their target insects have high rates of resistance.

Synthetic insecticides:

World Health Organization has approved a list of synthetic insecticides which are used commercially to treat adult mosquitoes to date. They have been categorized under four different classes which include organochlorines (now banned in most countries), organophosphates, carbamates and pyrethroids (Zaim and Guillet, 2002). Only a limited number of insecticide classes are available for adult mosquito control. No new malaria mosquito adulticide has been approved by the WHO in the last 15 years (Nauen, 2007). It is important to note that these four chemical classes of insecticides possess only two different modes of action indicating less target diversity when compared to agricultural pesticides (Nauen and Bretchneider, 2002). Moreover, the use of chemical insecticides results in undesirable effects such as increased physiological resistance in vectors, environmental pollution lead to bio-amplification in food chain and killing of non-target populations such as earthworms, birds etc.

Insecticide resistance:

Resistance is defined as the developed ability in a strain of insects to tolerate doses of toxicant which would prove lethal to majority of individuals in a normal population of the same species (WHO,1957). The resistance to insecticides is considered to be a recent evolutionary adaptation in insects which occurs in less than one century with response to sequential application of insecticides. The possible mechanisms to develop resistance is to enhance the ability of insects to detoxify the insecticide molecules and to alter the target sites so that insecticide molecules no longer bind with the action sites (Brattsten, 1986).

The emergence of resistance act as major hurdle in the line of current vector control programmes. More than 40 years of intensive synthetic insecticides use to control arthropod pests and disease vectors have resulted in pesticide resistance among over 450 species (Georghiou, 1986). Resistance is commonly monitored by bioassay either by determining LC50 value or by using uniform diagnostic doses (Feng Cui *et al.,* 2006). Susceptibility studies of malaria vectors *A.stephensi Liston* and *A.subpictus Grassi* , collected from different locations in arid and semi-arid regions of India are conducted by adulticide bioassay of DDT, malathion, deltamethrin and larvicide bioassay of fenthion, temephos, chloropyriphos using diagnostic doses. Both the *Anopheline sp*. showed variable

resistance to DDT and malathion, larvae of *Anopheline sp.* showed resistance to chloropyrophos followed by fenthion (Tikar *et al.,* 2011). In recent years, the knowledge of resistance status is essential to select a particular insecticide against target species in vector control programs. Hence, Worldwide Insecticide resistance Network (WIN, http://win-network.ird.fr) collaborates with internationally recognized institutions in vector research to track insecticide resistance at a global scale. The objective of WIN is to provide WHO and member states to evidence and expertise resistance management and deployment of alternative arbovirus vector control measures (Corbel *et al.,* 2016). In order to control human disease vectors, there is a need for alternate, more effective and environment-friendly control agents which enable long term sustainable results.

Biological agents:

The balance of nature depends to a large extent on the regulation of population densities by parasitoids, predators, competitors, parasites and pathogens. These natural enemies play an important role in checking the proliferation of vectors in nature. In this aspect various biological control agents have been thoroughly investigated with the support of World Health Organization Special Programme for Research and Training in Tropical Diseases(WHO/TDR) (Mulla, 1990). A large number of mosquito pathogens and parasites have been isolated and studied for the bio control of mosquitoes.The term biological control is defined as the control of pests, including the vectors of human disease by the direct or indirect use of natural enemies with or without their metabolites(WHO, 1982).The first biocontrol was *Bacillus popilliae*, entomopathogenic bacteria which was used against larvae of Japanese beetle. It was the first bacterium registered as insecticide in United States (Zhang *et al.,* 1997). Processed formulations were applied into soil and the pest population remains suppressed for more than 10 years after one application.

Entomopathogenic Bacteria:

Insect pathogenic bacteria are present in the families Pseudomonadaceae, Enterobacteriaceae, Lactobacillaceae, Micrococcaceae and Bacillaecae. In the past few decades, several bacterial isolates and strains of spore forming bacteria have been isolated that produce parasporal proteins which show high toxicity against insects (Katara *et al.,* 2012). Based on the safety to non-target organisms, only members of Bacillaceae (Order: Eubacteriales) were the most studied, commercialized, and successfully used in microbial control of lepidopteron, dipteran and coleopteran insect pests (Lacey *et al*.,1986). Among these, two bacteria such as *Bacillus thuringeinsis serovar israelensis (Bti)* and *Bacillus sphaericus (Bs)* have been successfully tested against mosquito larvae. There are certain guidelines for laboratory and field testing of mosquito larvae and it remain as universal method to test any biocontrol agent (WHO, 2005).

Bacillus thuringiensis israelensis(Bti):

Bacillus thuringiensis is a gram positive, rod shaped, spore forming bacterium characterized by its ability to exhibit insecticidal properties. This bacillus crystalline inclusions dissolve in the larval midgut, releasing one or more insecticidal crystal proteins (also called delta endotoxins) of 27 to 140 kilodaltons(kDa) (Hofte and Whitely, 1989). This appears to be a synergistic interaction between four proteins resulting in a highly complex mode of action which lead to the toxicity of mosquito larvae and with no resistance development. The *Bti* spores and parasporal crystals must be ingested by the larval (feeding) stage of target organism to cause mortality. The toxin binds to a receptor on the midgut cell wall resulting in pore formation in the cell and leading to death of the larva. *B. thuringiensis* was found to induce cellular and oxidative stress prior to mosquito death (Ahmed, 2013).

Bti is highly pathogenic against mosquitoes (*Culicidae*) and black flies (*Simuliidae*) and has some virulence against certain other *Diptera* especially *Chironomidae (*midges). Previouly, formulations of *Bacillus thuringiensis* have been used successfully as biocontrol agent to control agricultural pests, but their role in control of dipteran species was recognized only after the discovery of *B.thuringiensis* serovar *israelensis (Bti).* In 1975-76 under a World Health Organization sponsored project, a new *Bt* strain was discovered in Israel by Goldberg and Margalit (1977). This strain was isolated from *Culex* sp. dead larvae mosquito. Later, it was identified as *Bt israelensis*, serotype H14 according to its flagellar antigenicity. As a result of extensive research on the efficacy and evaluation of agents, *B.thuringiensis* (H-14) was effective in the field and registered for mosquito control in 1980 (Mulla *et al.,* 1984).

Table 1: List of *Bacillus thurigiensis israelensis* strains reported as mosquitocidal bacterial strains

Bacillus thuringeinsis israelensis	**Selected references**	**Source of isolation**
B.t jegathesan	Seleena and Lee,1990; Seleena *et al.,*1995	Malaysia(soil)
B.t medellin	Orduz *et al.,*1992; 1996; Thiery *et al.,*1996,	Colombia(soil)
B.t jegathesan	Seleenaand Lee,1990, Seleena *et al.,*1995	Malaysia(soil)
B.t sotto	Ohba *et al.,* 2000; Ohugushi *et al.,*2003	Okinawa, Japan(soil sample)
B..t. fukuokaensis	Ohba and Aizawa,1990; Lee and Gill, 1997; Guerchicoff *et al.,*1997	Japan
B.t.kyushaensis	Ohba and Aizawa, 1979; Held *et al.,*1990	Japan (*B.morri* breeding site)
B.t.israelensis	Goldberg and Margalit,1977	Israel(sewage pond)
B.t. morrisoni	Padua *et al.,* 1984	Phillippines(soil sample)
B.t. darmastadensis	Padua *et al.,*1980,	Japan
B.t. canadensis	Ishi and Ohba,1993	Iraq(soil)
B.t. thompsoni	Manonmani and Hoti,2001	India(soil)

More than 40,000 species of *Bacillus thuringiensis* have been isolated and identified which belongs to 39 serotypes*.* These include strains with various serotypes such as *Bt.canadensis*, *Bt.thompsoni*, *Bt.malaysiensis* and *Bt.jegathesan*. Among these, *Bt.medellin* and *Bt.jegathesan* appear as good candidates for further characterization and investigation (Ragni *et al.,* 1996).

These organisms are active against either *Lepidoptera*, or *Diptera* or *Coleoptera. Bti* was found to be specific toxic to larvae of 109 mosquito species *Bti* has an LC50 in the range of 10–13 ng/ml against the fourth instar of many mosquito species (Federici *et al.* 2003). Generally, *Culex* and *Aedes* are highly susceptible compared to *Anopheles* which are less susceptible (Balaraman *et al.,* 1983). Much higher concentrations of *Bti* are required to induce mortality in anopheline larvae than in *Aedes* species.

Limitations:

B.t.i formulations produced commercially are not active against adult flies, though the proteins in the parasporal body can be able to destroy the midgut epithelium of adults. This is mainly due to inability of proteins to penetrate the cuticle. There are no available methods to induce adult flies to ingest formulations under field conditions. Therefore, *Bti.* formulation commercially available at present are used as larvicides not as adulticides. It is well known that the toxicity of *Bti* lasts only a few days at most and efficacy can be reduced within 24 hours (Becker *et al.,* 1993). In addition, *Bti* does not survive long in highly polluted water and is particularly prone to UV light inactivation in strong sunlight (Mulla, 1990).

Most of these formulations offer high levels of initial control, but with very little residual activity. This has necessitated weekly application of these formulations to keep the larval population under constant check, which would increase logistics and cost (King *et al.,*1997). Therefore, a new formulation with long residual life and new mode of action is necessary. Due to high cost of production compared to chemical pesticides, operational success of *Bti* against the three major vectors is only limited to temperate regions (Europian countries) of the world, where these vectors are considered as nuisance pests (Porter *et al.,* 1993).

Bacillus sphaericus(Bs):

Bacillus sphaericus is another most extensively studied spore forming bacterium for its mosquitocidal properties. During sporulation, the active strains produce crystal toxin which is a binary toxin. The 51 and 42 kDa mosquitocidal crystal proteins of *B.sphaericus* are unique among bacterial insect toxins which have a low sequence similarity and are distinct from all of the cloned and sequenced insect toxins of *Bacillus thuringiensis* (Baumann *et al.,* 1991). Upon ingestion of this toxin by mosquitoes, they bind to specific receptors present in the midgut brush-border membrane and cause damage to midgut cells and lead to death. The first reported *Bacillus sphaericus* was not effective strain but after the isolation of *B.s* from

Indonesia (strain 1593) which is highly mosquitocidal (Charles *et al.,* 1996).Currently nine serotypes are known to contain active strains of *Bacillus sphaericus*. *Bacillus subsp isrealensis* and *B.sphaericus* differ in the nature of toxin and their host range.

In general, *B.sphaericus* is more effective against *Culex* spp and *Anopheles* but less effective to *Aedes* spp*. B.isrealensis subsp.* remain effective against *Aedes and Culex* spp but not to *Anopheles* spp *(*Lacey and Undeen,1986, Mulla, 1990*)*. In addition, *B. sphearicus* has its ability to survive in polluted aquatic environments but *B.ti* used to lose its ability in that environment (Mulla *et al.,* 1984, Davidson *et al.,1984,).* Most of mosquitocidal *B.s* strains were isolated successfully for the past 30 years. The most active strains 1593 and strain 2362 which belong to serotype 5a5b (Charles *et al.,*1996, Delecluse *et al.,*2000). *Bacillus sphaericus* VCRC-B547 isolated from excreta of arid birds has shown higher toxicity against *Cx.quinquefasciatus, An.stephensi* and *Aed.aegypti* (Poopathi *et al.,* 2014). *Bs* tend to have higher residual activity than *Bti* in polluted waters. As a result, commercial product Vectolex (Abbott Laboratories) based on strain 2362 is marketed in many countries especially in polluted aquatic environments*.*

Limitations:

Though, *Bacillus sphaericus* remain effective against *Culex* spp., repeated application in the field for long term effect will lead to development of resistance in target species. Persistance and recycling potential of *B. sphaericus* are more achievable in polluted than in clear waters (Mulla *et al.,* 1984). Because, recycling is important phenomenon where toxin production continues during a period where several generations of target species are produced.

The lower sensitivity of *Bs* may result from the fact that the protein of the bacterium is enclosed in the exosporium, whereas the delta endotoxin of *Bti* is uncoated. It is possible that coated spore-crystal complex is more tolerant to UV light than the uncoated protein. This feature is also responsible for the slow mode of action of products based on *Bs* and its potential to persist under certain field conditions (Lacey, 2007). The biolarvicide formulation from *Bs* strain is reported to be less effective against *Anopheles culicifacies* and hardly effective against *Aedes aegypti (*Mittal*,* 2003). *Bs* is at high risk of selecting resistance

Table 2: Commercially available *Bacillus sphaericus* strains as mosquito larvicides.

Strain number	source of isolation	References
2297	Sri Lanka	Wickremesinghe RSB and Mendis CL, 1980
1593	Nigeria	Weiser J, 1984
2362	China	Liu EY *et al.,* 1989
VCRC-B547	Pondicherry	Poopathi *et al.,* 2014

Table 3 : List of *Pseudomonas* species reported as mosquitocidal bacterial strains

Pseudomonas species	Source of isolation	References
Pseudomonas fluorescens	Dead mosquito larva	Murty *et al.,*1994; Prabakaran *et al.,2003;* Sadanandane *et al.,* 2003; Prabakaran *et al.,* 2009; Prabakaran *et al.,* 2015; Pushpanathan and Selvaraj Pandian, 2008; Varun Rajan and Selvaj Pandian *et al., 2008,*Usharani and Paily, 2014; Lalithambika *et al.,* 2014; Athisayamary *et al.,* 2015; Mahamuni *et al.,* 2015;
Pseudomonas pseudomallei	Soil samples (Malaysia)	Lee and Seleena,1990.
Pseudomonas aeroginosa	*Guppies(Poectilia reticulate)*	Lysenko and Kuchera, 1968; Chadde,1992, De Barjac, 1989.
Pseudomonas frederiksbergiensis	contaminated soil (Saudi Arabia)	Ahmed *et al.,* 2014; 2015

in mosquito population. In fact, resistance to *B.s* has already been reported in field populations of *Culex* spp in China, Brazil, France and India (Sinegre *et al.,* 1994, Rao *et al.,* 1995, Silva Filha *et al.,* 1995,Yuan *et al* , 2000) with resistance levels in some areas of China reported as >20,000 fold. The potential key strategy for delaying resistance to mosquitocidal proteins is to use mixture of toxins that act at different targets within the insects (Writh *et al.,* 2005)

Resistance against *Bti* and *Bs*:
Due to continuous selection pressure and cross resistance, mosquito populations develop resistance against *Bs* binary toxin (Bin) both in the laboratory and field trials (Sinegre *et al.,* 1994). In Brazil, it was reported that the tenfold increase in resistant population found in open drains and covered cessepits in a small area where all the breeding sites were treated during two year period with a total of 37 treatments (Wirth *et al.,* 2000). But in case of *Bti* strains, they have been used for mosquitoes and Black flies for about 20 years, yet no resistance to this bacterium has been reported. In contrast to their sub species only low levels of resistance was observed in the laboratory experiments. The reason behind is selection of *Culex quinquefasciatus* with mutants of *B.thuringiensis sub species israelensis* that contained different combinations of its Cry proteins and Cyt1Aa delayed the evolution and expression of resistance to mosquitocidal Cry proteins (Wirth *et al.,* 2005)

Recombinant bacterial strains for vector control:
Commercial products such as VectoBac and Teknar based on *Bacillus thurigiensis subsp. israelensis(Bti),* VectoLex based on *Bacillus sphaericus* are most widely used as vector control products. Even though these products gain commercial success in developed countries but their high cost of fermentation, limited their use in developing countries. Lack of persistence due to settling of the spore-crystal complexes and narrow host range compared with chemical insecticides limited the usage of wild strains of *Bti* and *Bs* (Ohana *et al.,* 1987). Recombinant DNA technology pave the way for enhancing the synthesis of mosquitocidal proteins and by enabling new endotoxin combinations from different bacteria to be produced within single strain (Federici *et al.,* 2003). Recombinant *Bti* able to produce Cyt1A, Cry proteins and Bs binary toxin, in which Cyt1A delays resistance to insecticides (Wirth *et al.,* 2005). Higher specificity, environmental safety of the recombinants compared to synthetic insecticides with increased efficacy will provide these novel strains to be used in the future pest and vector control programmes (Park and Federici*,* 2009).

Clostridium bifermentans serovar Malaysia:
The first anaerobic mosquitocidal isolate, CH18 was isolated and identified from Mangrove swamp soil from Malaysia. Hence it was named as *C.bifermentans serovar Malaysia(Cbm)*.Another strain was isolated from the forest and reported as *C.bifermentans serovar pariba(Cbp)*(Seleena *et al.,* 1997).Both these strains were active against *Anopheles* larvae and in increasing level of susceptibility to *Aedes* and *Culex* species. Their toxicity remains similar to *Bti* strains but the toxic factors are different from *Bt.* Though the Clostridium species includes human pathogens, the safety of *Cbm* strains as potential bioinsecticide is highly considerable (Thiery *et al.,* 1992).

Pseudomonas species:
Pseudomonas species show remarkable and physiological versality, enabling colonization in diverse terrestrial and aquatic habitats (Palleroni, 1992). They are generally aerobic, gram-negative bacteria, ubiquitous in agricultural soils and are well adapted to grow in the rhizosphere. Stainer *et al., (*1966) conducted a fundamental study on the pseudomonas that result in an extensive phenotypic characterization in which the genus was subdivided into species and species into groups. *Pseudomonads* possess many traits that make them well suited as biocontrol and growth-promoting agents. Many biocontrol agents from *P. fluorescens* are well characterized for their ability to produce antimicrobial compounds, including 2,4-diacetylphloroglucinol (DAPG), phenazines, hydrogen cyanide and surfactants (Haas and De´fago, 2005).

Some exotoxins such as *Pseudomonas aeruginosa Migula* have been noted to be absorbed through the cuticle of insects and act on the haemolymph proteins. Exotoxins of microbial origin, including *Pseudomonas species* are also known to be toxic to larvae of mosquitoes as well as lepidopteran insects (Murty *et al.,* 1994).

The larvicidal effects of the culture supernatants of *Pseudomonas fluorescens*(MSS-1), originally isolated from deceased mosquito larvae reported to be active against *Culex quinquefasciatus, Anopheles stephensi,*

Aedes aegypti. A microbial formulation of *Pseudomonas fluorescens* (VCRC 426) was developed and formulated and tested against 4th instar larvae and pupae of three major vectors. *A. stephensi* was found to be most susceptible followed by *Culex quinquefasciatus* and *Aedes aegypti.* This was the first report that exotoxin remain effective against the pupae of the three species of mosquitoes at a very low concentration that of larvae (Prabhakaran *et al.,*2002). Field valuation of VCRC B426 formulation of *P.fluorescens* against *Culex quinquefasciatus* larvae and pupae showed 100% elimination of larvae and pupae at day1 after treatment and 80% reduction in pupal density (Sadanandane *et al.,*2003). The exotoxins produced by *Pseudomonas fluorescens* exhibited marked larvicidal and pupicidal activity against *A. aegypti and A. albopictus* (Pushpanathan and Selvaraj Pandian, 2008).

Binding of *Pseudomonas fluorescens* proteins to specific receptors plays an important role in the mode of action. It has been reported that binding of mosquitocidal proteins to the midgut region of treated larvae and pupae leads to considerable increase in the marker enzyme activity and Cytochrome C oxidase activity in th treated *Aedes albopictus* cell lines (Usharani and Paily, 2014). *Pseudomonas fluorescens Migula* (VCRCB426) produces secondary metabolite which is analysed and found as rhamnolipid. It is reported as first mosquito pupicidal compound which is found active against *culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* (Prabakaran *et al.,* 2015). The major limitation of pseudomonads as biocontrol agents is their inability to produce resting spores which remain problematic in formulation of the product. Most of commercial products of *Bti* and *Bs* have their spore-crystal complex which have longer storage facility.

CONCLUSION

Collectively, arthopods are responsible for the transmission of vector-borne diseases both in human and animals. Over the past 30 years, there has been a global re-emergence of infectious diseases particularly vector-borne diseases with an increased frequency of epidemic transmission and expanding their geographical distribution. Many factors directly or indirectly contribute to emergence of vector borne diseases recently. Distribution of these diseases is determined by a complex dynamic of environmental and social factors such as globalization of travel and trade, unplanned urbanization, climate change etc which are having a significant impact on these diseases transmission in recent years These include climate change pattern, global trade, rapid unplanned urbanization, socioeconomic status, vector control programs which are highly influencing the current vector diseases epidemiology (Gubler, 2009). It has also been reported that the vectors in several countries has developed resistance to most of the highly effective class of insecticides. Hence, there remains a great challenge to control vector borne diseases. It is essential to develop a novel bioinsecticide which posses new mode of action, rapidly kills target species, high specificity and with commercial value. It is also essential to review about biocontrol agents for vector control and under laying fundamental capacities including technical expertise, stronger surveillance systems and better laboratory infrastructure facilities (WHO, 2014).

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Biocontrol efficacy of selected soil microbes

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CERTIFICATE

This is to certify that the thesis entitled "**Biocontrol efficacy of selected soil microbes**" submitted by **Mrs. Sivakami .V** for the Degree of Doctor of Philosophy in Science of Madurai Kamaraj University, Madurai is based on the results of studies carried out by her under our guidance and supervision. This thesis or any part thereof has not been submitted elsewhere for the award of any other degree or diploma.

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DECLARATION

I hereby declare that the research work presented in this thesis entitled "**Biocontrol efficacy of selected soil microbes"** has been originally carried out by me under the guidance and supervision of **Dr. S. Kannan**, Professor & Head, Research Guide & Supervisor, Department of Environmental Studies, School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai, India. This work has not been submitted elsewhere for the award of any other degree or diploma.

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Sivakami.V

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ABBREVIATIONS

µl	microlitre
mg	milligram
g	gram
nm	Nanometer
^{0}C	degree Celsius
ml	milliliter
hrs	hours
mins	minutes
pH	hydrogen potential
EC	Electrical Conductivity
APHA	American Public Health Association
CFU	Colony Forming Unit
DNA	deoxyribonucleic acid
PCR	Polymerase Chain Reaction
EDTA	Ethylenediamine tetraacetic acid
Bp	base pair
BLAST	Basic Local Alignment Search Tool
MEGA	Molecular Evolutionary Genetics Analysis
KDa	Kilo Daltons
rpm	rotation per minute
ppm	parts per million
SEM	Scanning Electron Microscope
$FeSO_4$	Ferrous sulphate

BSA	Bovine Serum Albumin
SRBC	Sheep Red Blood Cells
SPSS	Statistical Package for Social Sciences
TAE	Tris-acetate-EDTA
PBS	Phosphate Buffer Saline
GPS	Glucose Peptone Salt
OD	Optical Density
HMDS	Hexamethyldisilazane
M	molarity